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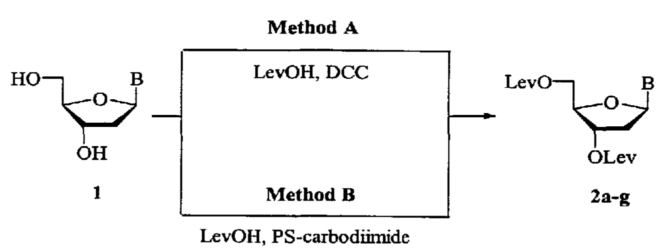
[Continued on next page]

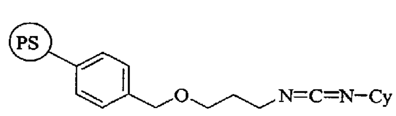
(54) Title: BUILDING BLOCKS FOR THE SOLUTION PHASE SYNTHESIS OF OLIGONUCLEOTIDES

(57) Abstract: The present invention is directed to methods for the preparation of 3' *0* and 5' *0*- levulinyl nucleosides from common precursors using an enzymatic approach.

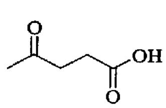


a, B= T; **b**, B= C; **c**, B= C^{Bz}; **d**, B= A; **e**, B= A^{Bz}; **f**, B= G; **g**, B= G^{iBu}

Method A: LevOH, DCC, DMAP, Et_3N, 1,4-Dioxane.
Method B: LevOH, PS-carbodiimide, DMAP, DMAP•HCl, Et_3N, 1,4-Dioxane.



PS-carbodiimide

LevOH= 

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BUILDING BLOCKS FOR THE SOLUTION PHASE SYNTHESIS OF OLIGONUCLEOTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Patent Application serial no. 09/822,903, filed March 30, 2001, the contents thereof being expressly incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to methods for the preparation of 3'-*O* and 5'-*O*-levulinyl nucleosides from common precursors using an enzymatic approach. These methods are useful for the large-scale synthesis of oligonucleotides.

BACKGROUND OF THE INVENTION

It is well known that most of the bodily states in mammals, including most disease states, are affected by proteins. Such proteins, either acting directly or through their enzymatic functions, contribute in major proportion to many diseases in animals and man. Classical therapeutics has generally focused on interactions with such proteins in efforts to moderate their disease causing or disease potentiating functions. Recently, however, attempts have been made to moderate the actual production of such proteins by interactions with molecules that direct their synthesis, such as intracellular RNA. By interfering with the production of proteins, it has been hoped to affect therapeutic results with maximum effect and minimal side effects. It is the general object of such therapeutic approaches to interfere with or otherwise modulate gene expression leading to undesired protein formation.

One method for inhibiting specific gene expression is the use of oligonucleotides and oligonucleotide analogs as "antisense" agents. The oligonucleotides or oligonucleotide analogs complimentary to a specific, target, messenger RNA (mRNA) sequence are used. Antisense methodology is often directed to the complementary hybridization of relatively

short oligonucleotides and oligonucleotide analogs to single-stranded mRNA or single-stranded DNA such that the normal, essential functions of these intracellular nucleic acids are disrupted. Hybridization is the sequence specific hydrogen bonding of oligonucleotides or oligonucleotide analogs to Watson-Crick base pairs of RNA or single-stranded DNA. Such base pairs are said to be complementary to one another.

Oligonucleotides and oligonucleotide analogs are now accepted as therapeutic agents holding great promise for therapeutics and diagnostics methods. But applications of oligonucleotides and oligonucleotide analogs as antisense agents for therapeutic purposes, diagnostic purposes, and research reagents often require that the oligonucleotides or oligonucleotide analogs be synthesized in large quantities.

Three principal methods have been used for the synthesis of oligonucleotides. The phosphotriester method, as described by Reese, *Tetrahedron* 1978, *34*, 3143; the phosphoramidite method, as described by Beaucage, in *Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs*; Agrawal, ed.; Humana Press: Totowa, 1993, Vol. 20, 33-61; and the *H*-phosphonate method, as described by Froehler in *Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs* Agrawal, ed.; Humana Press: Totowa, 1993, Vol. 20, 63-80.

The phosphotriester approach has been widely used for solution phase synthesis, whereas the phosphoramidite and *H*-phosphonate strategies have found application mainly in solid phase syntheses. Recently, Reese reported a new approach to the solution phase synthesis of oligonucleotides on *H*-phosphonate coupling. *See*, Reese *et al. Nucleic Acids Research,* 1999, *27,* 963-971, and Reese *et al. Biorg. Med. Chem. Lett.* 1997, *7,* 2787-2792, which is incorporated herein by reference. Solution phase synthesis is the method of choice in producing large-scale quantities of oligonucleotides.

These solution phase methods require the use of nucleoside monomer building blocks bearing protecting groups on the 3'-O and/or the 5'-O positions. The protecting groups should be stable to coupling conditions and selectively cleaved without affecting other protecting groups in the molecule. One such protecting group is the levulinyl group, $-C(O)-(CH_2)_2-C(O)-CH_3$. However, the preparation of nucleosides bearing these protecting groups involves several tedious chemical protection/de-protection and/or purification steps.

For example, the 3',5'-di-*O*-levulinyl protection of nucleosides can be accomplished using a well-established method wherein nucleosides are selectively acylated at their hydroxyl

sites by reacting the nucleosides with levulinic acid in the presence of DCC (dicyclohexylcarbodiimide). Despite the utility of this method, it suffers from at least one significant problem. The method requires a large excess of DCC to achieve optimal yields. The excess DCC is converted to DCU (dicyclohexylcarbodiimide) upon completion of the reaction, which must be separated from the reaction mixture. Unfortunately, for large-scale syntheses, the separation step requires considerable time and expense.

Prior to the present invention, synthesis of 5'-O-levulinyl nucleosides was accomplished by reacting parent nucleosides with levulinic acid and 2-chloro-1-methylpyridinium iodide. Iwai *et al.*, *Nucleic Acids Res.* 1988, *16*, 9443-9456; Iwai *et al.* T*etrahedron* 1990, *46*, 6673-6688. However, because this method does not afford selective acylation of the 5'-hydroxyl function, additional purification and deprotection steps are necessary because both 3'-acyl and 3',5'-diacyl derivatives are formed in the reaction. After the 3',5'-diacyl derivatives are separated by chromatography, the residue must be treated with DMTrCl to remove the 3'-acyl compound. Finally, an additional purification by chromatography isolates the 5'-*O*-levulinyl derivatives in very low yields.

Before now, the synthesis of 3'-*O*-levulinyl nucleosides (2'-deoxy or 2'-protected) was accomplished by the treatment of parent nucleosides with levulinic acid or levulinic anhydride and DCC. One of the major drawbacks of this method is that it requires that the 5'-hydroxyl function be protected as a 5'-*O*-DMTr group prior to acylation with levulinic acid. The 5'-*O*-DMTr group must then be removed in an acid medium to afford the 3'-*O*- protected nucleosides. *See*, Reese *et al.*, *Nucleic Acids Res.* 1999, *27*, 963-971, and Reese *et al.*, *J. Chem. Soc., Perkin Trans. 1* 1999, 1477-1486.

Commercially viable methods for the large-scale synthesis of oligonucleotides are constantly being explored. It has been found that the application of biocatalysts in organic synthesis has become an attractive alternative to conventional chemical methods. *See*, Carrea, *et al. Angew. Chem. Int. Ed.* 2000, *39*, 2226-2254; Bornscheuer, *et al. Hydrolases in Organic Synthesis. Regio- and Stereoselective Biotransformations*; Wiley-VCH: Weinheim, 1999. Enzymes catalyze reactions with high chemo-, regio-, and stereoselectivity. *See*, Ferrero *et al. Chem. Rev.* 2000, *100*, 4319-4347; Ferrero *et al.*, *Monatsh. Chem.* 2000, *131*, 585-616. It has previously been reported that *Candida antarctica* lipase B (CAL-B) catalyzes acylation at the 5'-hydroxyl group of nucleosides with high selectivity. *Pseudomonas cepacia* lipase (PSL) shows unusual regioselectivity towards the secondary alcohol at the 3'-position of 2'-

deoxynucleosides. Moris *et al.*, *J. Org. Chem.* 1993, *58*, 653-660; Gotor *et al. Synthesis* 1992, 626-628.

In the last few years the use of antisense oligonucleotides has emerged as an exciting new therapeutic paradigm. As a result, very large quantities of therapeutically useful oligonucleotides are required in the near future. In view of the considerable expense and time required for synthesis of oligonucleotide building blocks, there has been a longstanding effort to develop successful methodologies for the preparation of oligonucleotides with increased efficiency and product purity.

SUMMARY OF THE INVENTION

The present inventors have discovered methods and reagents that are useful in, for example, the large-scale synthesis of oligonucleotides. The methods of the present invention help to minimize the number of steps required to yield desired results using an enzymatic approach. In some embodiments, the present invention provides methods and reagents for synthesizing both 3'-*O*-levulinyl nucleosides and 5'-*O*-levulinyl nucleosides from a common precursor. In such embodiments, the regioselective deprotection of a 3',5'-di-*O*-levulinyl nucleoside yields either a 3'-O levulinyl nucleoside or a 5'-O-levulinyl nucleoside, depending upon parameters disclosed herein. In particular, either the 5'-*O*-levulinyl nucleoside or the 3'-*O*-levulinyl nucleoside can be selectively produced by contacting the corresponding 3',5'-di-*O*-levulinyl nucleoside precursor with a particular hydrolase, such as a lipase. Surprisingly, it has been found that the presence of selected hydrolases, such as lipases, in deprotection reaction protocols gives rise to regioselectivity of de-acylation at either the 3'- or 5'- position, depending upon the lipase that is chosen.

In other embodiments, the present invention provides a method of selectively preparing a nucleoside having a levulinyl at either the 3'-O or 5'-O position by contacting the nucleoside with an acylating agent for introducing the levulinyl group in the presence of a selected hydrolase (e.g. a lipase). Surprisingly, it has been found that selective acylation at either the 3'-O or 5'-O position of a nucleoside may be obtained, depending upon the specific hydrolase that is chosen to catalyze the reaction.

According to some embodiments, methods are provided for regioselectively deprotecting a 3',5'-di-*O*-levulinylnucleoside, said methods comprising selecting a hydrolase, e.g. a lipase, that is effective to direct a regioselective hydrolysis of one of the levulinyl

positions, without causing an undesired level of hydrolysis on the other of the levulinyl positions, and contacting the 3', 5'-di-*O*-levulinyl nucleoside with the lipase for a time and under conditions effective to yield either a 3'-*O*-levulinyl or a 5'-*O*-levulinyl nucleoside. Examples of hydrolases that are amenable to the present invention include the lipases *Candida antarctica* lipase B (CAL-B), *Candida antarctica* lipase A (CAL-A), *Pseudomonas cepacia* lipase (PSL), porcine pancreatic lipase, *Chromobacteriaum viscosum* lipase, *Mucor miehei* lipase, *Humicola lanuginosa* lipase, *Penicillium camemberti* lipase, *Candida rugosa* lipase, and others.

According to some embodiments, methods are provided for regioselectively acylating a nucleoside, said methods comprising selecting a hydrolase, such as a lipase, that is effective to direct a regioselective acylation of one of the -OH positions of the nucleoside, without causing an undesired level of acylation on the other of the levulinyl positions, and contacting the nucleoside with an acylating agent for introducing the levulinyl group and the selected lipase for a time and under conditions effective to yield either a 3'-*O*-levulinyl or a 5'-*O*-levulinyl nucleoside. Examples of lipases that are amenable to the present invention include *Candida antarctica* lipase B (CAL-B), *Candida antarctica* lipase A (CAL-A), *Pseudomonas cepacia* lipase (PSL), porcine pancreatic lipase, *Chromobacteriaum viscosum* lipase, *Mucor miehei* lipase, *Humicola lanuginosa* lipase, *Penicillium camemberti* lipase, *Candida rugosa* lipase, and others.

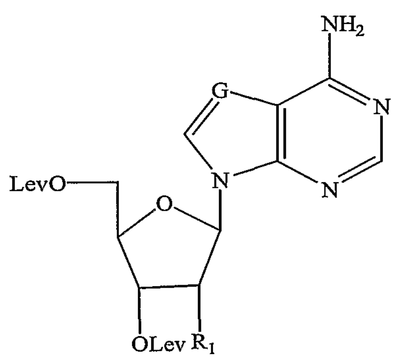
According to some embodiments of the present invention, a 3',5'-di-*O*-levulinyl nucleoside is deprotected at the 5'-O-levulinyl position by contacting the diprotected nucleoside with CAL-B for a time and under conditions effective to regioselectively hydrolyze the 5'-*O*-levulinyl position without affecting the 3'-*O*-levulinyl position.

According to further embodiments of the present invention, a nucleoside is selectively acylated at the 5'-OH position (or simply 5'- position) by contacting the nucleoside with an acylating agent for introducing the levulinyl group in the presence of CAL-B for a time and under conditions effective to regioselectively acylate the nucleoside at the 5'-position to form the 5'-*O*-levulinyl nucleoside.

In further embodiments, a 3'-, 5'- di-*O*-levulinyl nucleoside is deprotected at the 3'-*O* levulinyl position by contacting the diprotected nucleoside with CAL-A or PSL-C for a time and under conditions effective to regioselectively hydrolyze the 3'-*O*-levulinyl position without affecting the 5'-*O*-levulinyl position.

In further embodiments, a nucleoside is regioselectively acylated at the 3'-OH position by contacting the nucleoside with an acylating agent for introducing the levulinyl group in the presence of CAL-A or PSL-C for a time and under conditions effective to regioselectively acylate the nucleoside at the 3'-OH position to produce the 3'-*O*-levulinyl nucleoside.

In some embodiments of the present invention, methods are disclosed for regioselectively deprotecting a 3'-, 5'-di-*O*-levulinyl nucleoside at the 5'-*O*-levulinyl position wherein the nucleoside has one of the following formulas:



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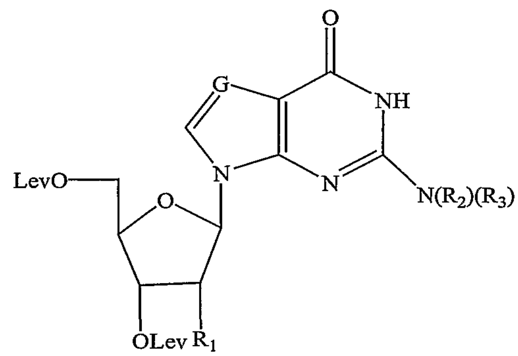

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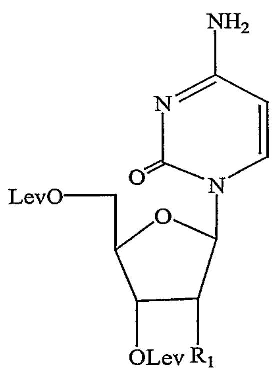

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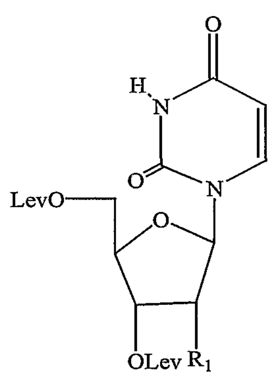

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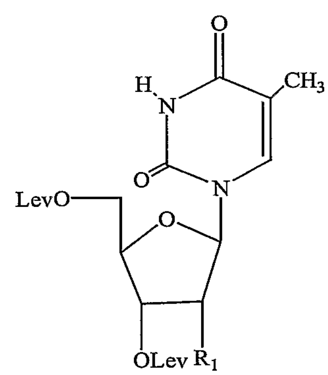


V

wherein:

R_1 is -H, -hydroxyl, a protected hydroxyl, a 2'-substituent or a 2'-protected substituent; and

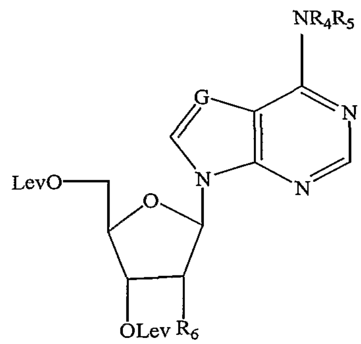
R_2 and R_3 are, independently, -H or an amino protecting group;

G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$, the levulinyl group;

the methods comprising selecting a lipase that is effective to direct a regioselective hydrolysis of the 5'-*O*-levulinyl position, without causing hydrolysis on the 3'-*O*-levulinyl position, and contacting the 3', 5'-di-*O*-levulinyl nucleoside with the lipase for a time and under conditions effective to yield a 3'-*O*-levulinyl nucleoside. A preferred lipase for 5'-*O*-levulinyl hydrolysis is CAL-B.

In still further embodiments, methods are provided for regioselectively deprotecting a nucleoside at the 3'-*O*-levulinyl position wherein the nucleoside has one of the following formulas:



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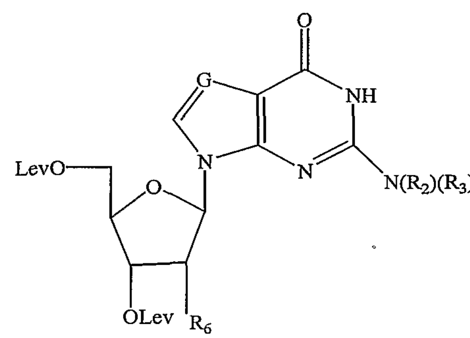

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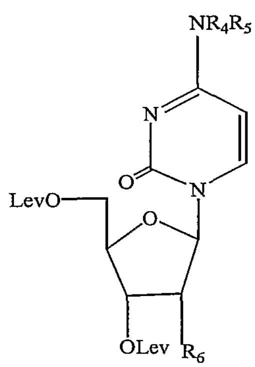

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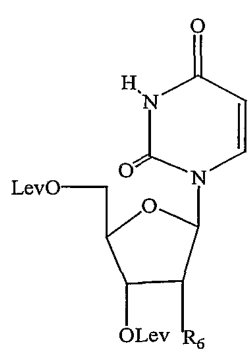

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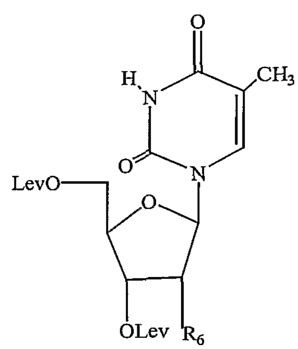


X

wherein:

R_6 is -H, -hydroxyl;

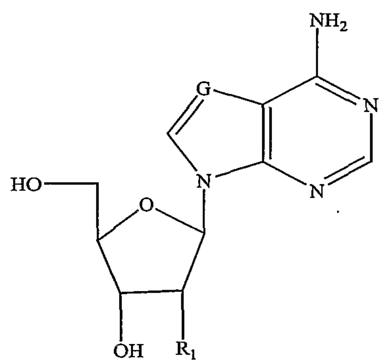
R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$;

the methods comprising selecting a lipase that is effective to direct a regioselective hydrolysis of the 3'-*O*-levulinyl position, without causing hydrolysis of the 5'-*O*-levulinyl position, and contacting the 3', 5'-di-*O*-levulinyl nucleoside with the lipase for a time and under conditions effective to yield a 5'-*O*-levulinyl nucleoside. Lipases that are preferable for hydrolysis at the 3'-*O*-levulinyl positions are, for example, CAL-A or PSL-C.

In some embodiments of the present invention, methods are disclosed for regioselectively introducing a levulinyl group in a nucleoside at the 5'-OH position wherein the nucleoside has one of the following formulas:



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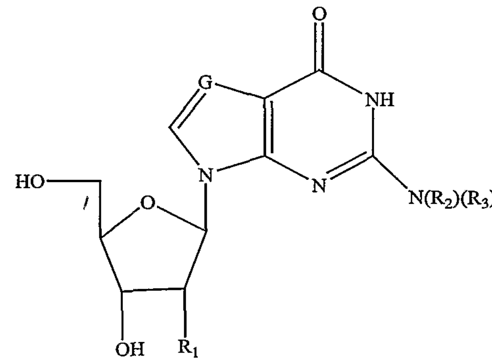

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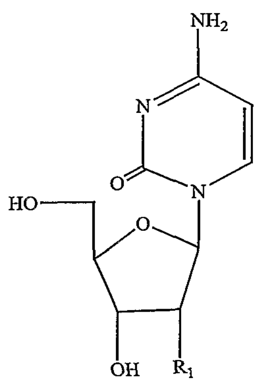

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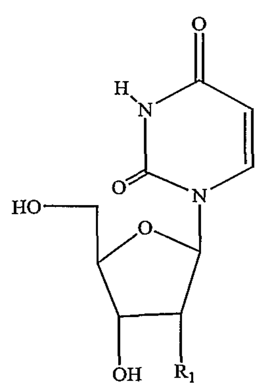

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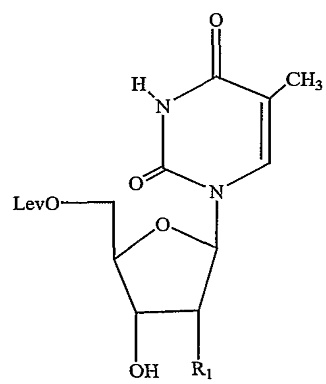


XV

wherein:

R_1 is -H, -hydroxyl, a protected hydroxyl, a 2'-substituent or a 2'-protected substituent; and

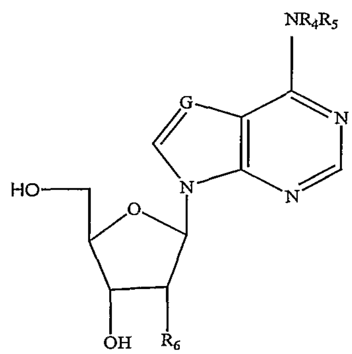
R_2 and R_3 are, independently, -H or an amino protecting group;

G is N or CH; and

Lev is -C(O)-$(CH_2)_2$-C(O)-CH_3, the levulinyl group;

the methods comprising selecting a lipase that is effective to direct a regioselective acylation of the 5'-O position, without causing acylation of the 3'-O position, and contacting the nucleoside with an acylating agent for introducing the levulinyl group and the lipase for a time and under conditions effective to yield a 5'-*O*-levulinyl nucleoside. A preferred lipase for 5'-*O*-levulinyl acylation is CAL-B.

In still further embodiments, methods are provided for regioselectively acylating a nucleoside at the 3'-OH position wherein the nucleoside has one of the following formulas:



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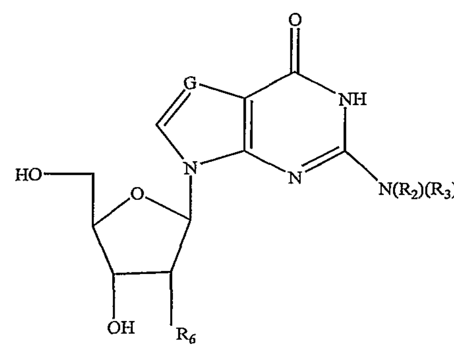

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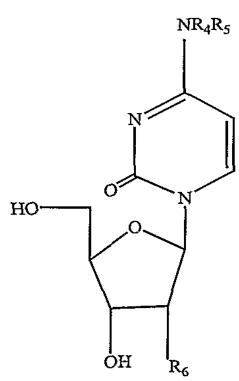

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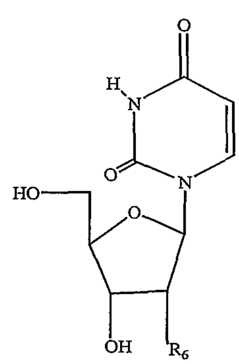

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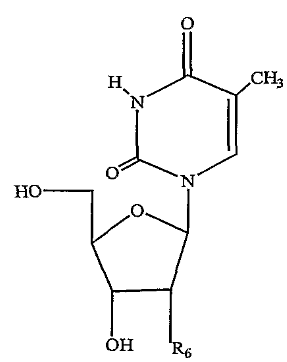


XX

wherein:

R_6 is -H, -hydroxyl;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

G is N or CH; and

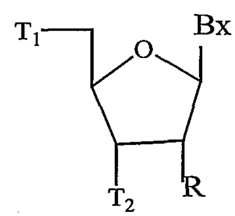
Lev is -C(O)-$(CH_2)_2$-C(O)-CH_3;

the methods comprising selecting a lipase that is effective to direct a regioselective acylation of the 3'-O position, without causing acylation of the 5'-O position, and contacting the nucleoside with an acylating agent for introducing the levulinyl group and the lipase for a time and under conditions effective to yield a 3'-*O*-levulinyl nucleoside. Lipases that are preferable for regioselective acylation at the 3'-O position are, for example, CAL-A or PSL-C.

In some embodiments of the present invention, methods are provided for acylating a hydroxyl moiety of a nucleic acid, such as a nucleoside or a nucleotide, at one or more of a 2'-O, 3'-O, or 5'-O position, said methods comprising reacting the nucleic acid with levulinic acid in the presence of a coupling agent, such as a carbodiimide, that is attached to a polymeric support for a time and under conditions effective to form an ester at the 2'-O, 3'-O or 5'-O position. Preferred polymeric supports comprise polystyrene or polyethylene glycol polymeric supports that are attached to cyclohexylcarbodiimide.

The present invention includes the esterification or acylation of any hydroxyl moiety, such as those found in carbohydrates or steroid molecules, by reacting the compounds containing the hydroxyl moiety with levulinic acid in the presence of a coupling agent that is attached to a polymeric support for a time and under conditions effective to form an ester between the hydroxyl moieties and the levulinyl group of the levulinic acid.

In some embodiments of the present invention, methods are provided for acylating at least one hydroxyl moiety on a compound having the following formula:



wherein:

B_X is a nucleobase;

T_1 and T_2 are, independently, -hydroxyl, a hydroxyl protecting group, an activated phosphate group, a nucleotide, a nucleoside, or an oligonucleotide;

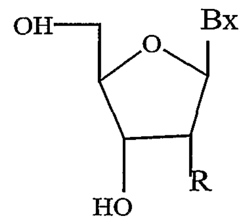
R is -H, -hydroxyl, a protected hydroxyl or a 2' substituent group;

provided that at least one of T_1, T_2 or R is -hydroxyl;

the methods comprising reacting said compound with levulinic acid in the presence of a coupling agent that is attached to a solid support, such as PS-cyclohexylcarbodiimide, for a

time and under conditions effective to form an ester between the hydroxyl moiety and the levulinyl group. In a preferred embodiment, T_1 and T_2 are -OH and R is -H or a 2'-substituent.

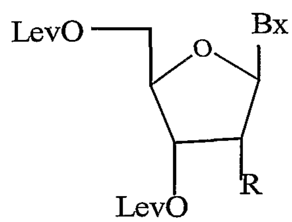
In preferred embodiments, methods are provided for acylating the 3'-O and 5'-O positions of a compound having the following formula:



wherein:

B_X is a nucleobase; and

R is hydroxyl or an optionally protected 2'-substituent comprising reacting said compound with levulinic acid in the presence of a coupling agent that is attached to a solid support for a time and under conditions effective to form a compound having formula:



wherein Lev is -levulinyl.

According to some embodiments of the present invention, methods are provided for generating a cyclohexylcarbodiimide derivatized polymeric support from a cyclohexylurea derivatized polymeric support, said methods comprising reacting the cyclohexylurea derivatized polymeric support with a dehydrating agent, such as tosyl chloride or $POCl_3$, in an organic solvent for a time and under conditions effective to yield the cyclohexylcarbodiimide derivatized polymeric support. In some embodiments, the organic solvent employed is CH_2Cl_2, $CHCl_3$, hexane, or pyridine.

In further embodiments of the present invention, methods are provided for generating a cyclohexylcarbodiimide derivatized polymeric support from a cyclohexylurea derivatized polymeric support, the methods comprising the steps of reacting the cyclohexylurea

derivatized polymer support with a dehydrating agent for a time and under conditions effective to form a salt and subsequently contacting the salt with an aqueous solution, such as aqueous NaOH, to form the cyclohexylcarbodiimide derivatized polymeric support.

BRIEF DESCRIPTION OF THE DRAWINGS

The numerous objects and advantages of the present invention may be better understood by those skilled in the art by reference to the accompanying detailed description and the following illustrative drawings, in which:

Figure 1 shows 3', 5'-di-*O*-acylation of a 2'-deoxynucleoside using levulinic acid and DCC or levulinic acid and PS-carbodiimide.

Figure 2 shows the enzymatic regioselective hydrolysis of a 3',5'-di-*O*-levulinyl 2'-deoxynucleoside.

Figure 3 shows the enzymatic regioselective hydrolyis of a 3',5'-di-*O*-levulinyl 2'-substituted nucleoside.

Figure 4 is a table depicting the results of regioselective hydrolysis of nucleosides 2a - 2g.

Figure 5 depicts the regioselective acylation of 2'-deoxynucleosides.

Figure 6 is a table depicting the results of regioselective acylation of 2'-deoxyribonucleosides 1a, 1c, le and 1g.

Figure 7 depicts the regioselective acylation of ribonucleosides.

Figure 8 is a table depicting the results of regioselective acylation of ribonucleosides 5a-5g.

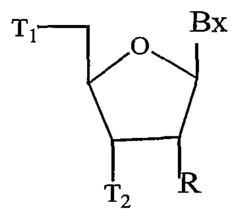
DETAILED DESCRPTION OF THE INVENTION

The present invention is directed to the preparation of nucleoside building blocks such as 3', 5'-di-*O*-levulinylnucleosides, 3'-*O*-levulinylnucleosides, and 5'-*O*-levulinylnucleosides that are especially useful in the large-scale synthesis of oligonucleotides.

According to some embodiments of the present invention, methods are provided for protecting a hydroxyl moiety of a nucleic acid at at least one of a 2'-O, 3'-O or 5'-O position comprising reacting the nucleic acid with levulinic acid in the presence of a coupling agent that is attached to a polymeric support for a time and under conditions effective to form an ester at the 2'-O, 3'-O or 5'-O position. The nucleic acids of the present invention include

nucleosides, nucleotides, oligonucleosides and oligonucleotides. In some embodiments, the nucleic acid is a nucleoside and the polymeric support is a polystyrene support or a polyethylene glycol support that is coupled to a coupling agent, such as cyclohexylcarbodiimide.

In preferred embodiments, the nucleic acid has the formula:



wherein:

B_X is a nucleobase;

T_1 and T_2 are, independently, hydroxyl, a protected hydroxyl, an activated phosphate group, a nucleotide, a nucleoside, or an oligonucleotide; and

R is -H, -hydroxyl, a protected hydroxyl, or a 2' substituent group;

provided that at least one of T_1, T_2 or R is -OH;

the method comprising reacting said compound with levulinic acid in the presence of a coupling agent that is attached to a solid support for a time and under conditions effective to form an ester between the hydroxyl moiety and the levulinyl group. In a preferred embodiment, T_1 and T_2 are -OH and R is H.

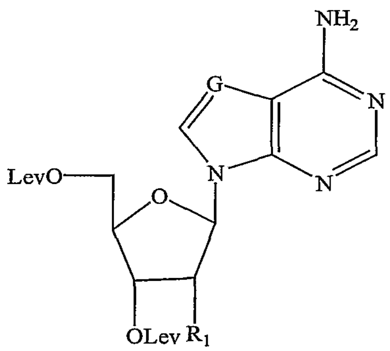
The protection methods of the present invention are not limited to acylation of the hydroxyl groups of nucleosides. Any hydroxyl functionality may be acylated using the methods of the present invention, including those found in carbohydrate or steroid molecules.

According some embodiments of the present invention, referring to Figure 1, 3', 5'-di-*O*-levulinyl nucleosides (2) were prepared from their corresponding natural nucleosides by treatment with levulinic acid and PS-carbodiimide in 1,4-dioxane in the presence of DMAP as a catalyst. Filtering off of the polystyrene beads removed the urea and the *N*-levulinylurea derivatives, which were polymer bound. 3', 5'-di-*O*-levulinylthymidine (2a) and 3',5'-di-*O*-levulinyl-2'-deoxyadenosine (2d) were isolated with 91% and 95% yield, respectively. The PS-dicarbodiimide, an expensive reagent, is recovered by reacting the cyclohexylurea derivatized polymer support with a dehydrating agent in an organic solvent. Preferred

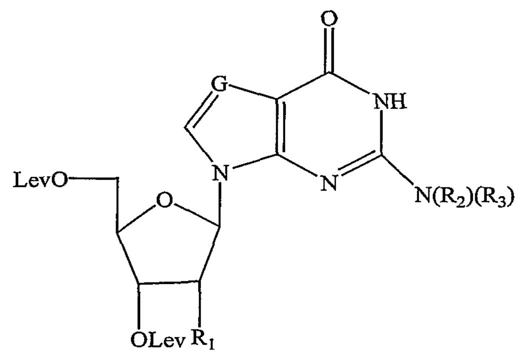
dehydrating agents include $POCl_3$ and tosyl chloride. Preferred organic solvents include CH_2Cl_2, $CHCl_3$, hexane, and pyridine.

Referring again to Figure 1, 3', 5'-di-*O*-levulinyl nucleosides can alternatively be prepared from the corresponding natural nucleosides (1) by treatment with 5.2 equivalents of levulinic acid (LevOH) and dicyclohexylcarbodiimide (DCC) in 1,4-dioxane in the presence of DMAP as catalyst. The reaction takes place through activation of the levulinic acid with DCC to obtain the *O*-acylurea intermediate. The excess of adduct evolves into the stable *N*-acylurea which was isolated like DCU as byproducts in the process. 3',5'-Dilevulinyl derivatives (2) were obtained in high yields (70-95%) after flash chromatography. The crude residue of the reactions was washed with Et_2O to eliminate the *N*-acylurea and subsequently dissolved in EtOAc from which the remaining DCU was separated by filtration. Almost quantitative yields were achieved for this acylation reaction. The level of purity was based on their ^{1}H-NMR which showed just traces of DCU and *N*-levulinylurea. Under these conditions no acylation was observed in the amino group of 2'-deoxyadenosine (1d) and 2'-deoxyaguanosine (1f). In the case of 2'-deoxycytidine (1b), a lesser amount of LevOH and DCC (3 equivalents) were used to minimize the formation of the aminoacyl derivative. As a consequence, longer reaction times were needed and some amount of the starting material remained unchanged. In spite of that, 68% isolated yield of 3',5'-di-*O*-levulinyl-2'-deoxycytidine (2b) were obtained after flash chromatography.

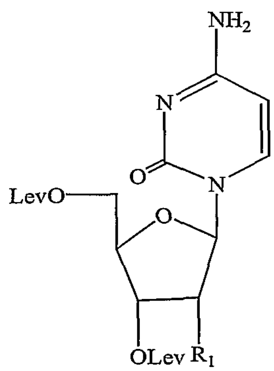
Regioselective deprotection of the common precursor, 3', 5'-di-*O*-levulinyl nucleoside, at the 5'-*O*-levulinyl position is effected by selecting a hydrolase, e.g. a lipase, effective to direct regioselective hydrolysis at the 5'-*O* -levulinyl position, without causing hydrolysis at the 3'-*O*-levulinyl position, and contacting the diprotected nucleoside with the lipase for a time and under conditions effective to hydrolyze the 3', 5'-di-O-levulinyl nucleoside at the 5'-*O*-levulinyl position. In some embodiments, the diprotected nucleosides have one of the following formulas:



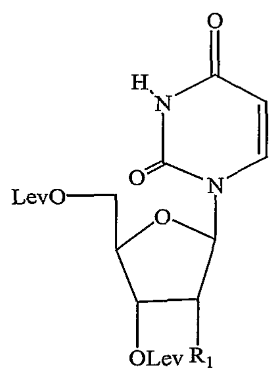
I



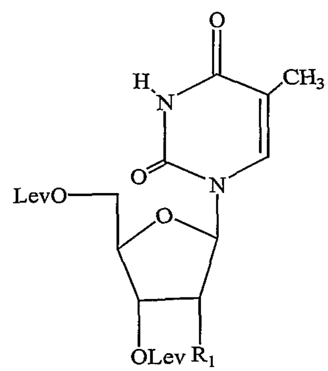
II



III



IV



V

wherein:

R_1 is -H, -hydroxyl, a protected hydroxyl, or a 2'-substituent; and

R_2 and R_3 are, independently, -H or an amino protecting group;

G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$.

For example, referring to Figure 2, a 3',5'-di-*O*-levulinylthymidine (2a) was treated with CAL-B at 40° C in 0.15M phosphate buffer (pH = 7) containing 18% of 1,4-dioxane. TLC showed total disappearance of the starting material after 62h (entry 1, Table 1). After usual workup, as described by Myers *et al. Trends Pharmacol. Sci.* **2000**, *21*, 19-23; Cook, *Nucleosides Nucleotides* **1999**, *18*, 1141-1162; Crooke, *et al. Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 107-129; and Matteucci *et al.* **1996**, *384*, 20-22, the contents of which are all incorporated by reference herein. ^{1}H-NMR spectra clearly indicated the selective hydrolysis of the 5'-levulinic ester and the presence of 3'-*O*-levulinylthymidine (3a) as unique product.

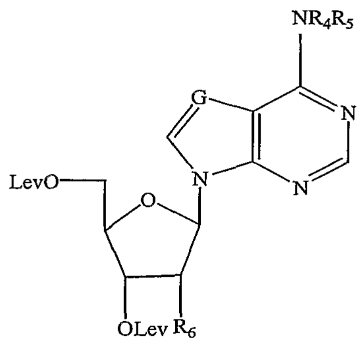
Traces of thymidine formed in the enzymatic reaction (showed by TLC) remained in the aqueous phase after extraction. Thus, pure compound (3a) was isolated with 85% yield.

Table 1, shown in Figure 4, also indicates that substrates 3',5'-di-*O*-levulinyl cytosine (2b), 3',5'-di-*O*-levulinyl adenosine (2d), and 3',5'-di-*O*-levulinyl-*N*-isobutylguanosine (2g) exhibit excellent selectivity towards the 5'-position, when hydrolyzed in the presence of CAL-B. The absence of the 5'-*O*-levulinyl derivative and the high yields with which the reactions take place are noteworthy. Also, in these cases, TLC showed traces of completely hydrolyzed nucleoside, which was easily removed with an aqueous extraction.

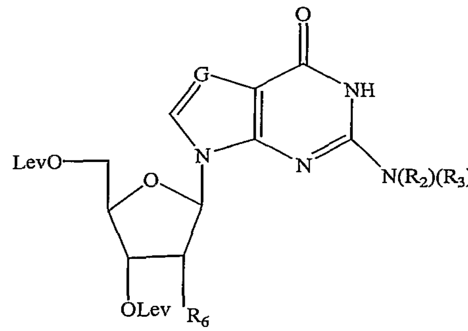
The hydrolysis reaction catalyzed by CAL-B on *N*-benzoyl-di-*O*-levulinyl-2'-deoxycytidine (2c) and *N*-benzoyl-di-*O*-levulinyl-2'-deoxyadenosine (2e) afforded *N*-benzoyl-2'-deoxycytidine (1c) and *N*-benzoyl-2'-deoxyadenosine (1e), respectively. Although several reaction conditions were tried, the process takes place without regioselectivity. It seems that the active site of CAL-B did not accommodate the *N*-protected adenosine and cytosine in the same manner as their unprotected counterparts. While not wishing to be bound to any particular theory, it is possible that the phenyl group could have some steric contact within the binding site, which may lead to unfavorable results.

2'-substituted nucleosides are also successfully selectively deprotected at the 5'-*O*-levulinyl position. Referring to Figure 3, all four nucleosides, 2'-methoxy-3', 5'-di-*O*-levulinyladenosine (6a), 2'-methoxyethoxy-3', 5'-di-*O*-levulinyladenosine (6b), 2'methoxy-3',5'-di-*O*-levulinyl-2'-deoxycytosine (6c), and 2'-methoxyethoxy-3', 5'-di-*O*-levulinyl-5-methyl cytosine (6d) were selectively hydrolyzed with CAL-B furnishing 7a-7d in high yields. The times and conditions effective to hydrolyze the nucleosides are not limited to those exemplified herein. Various times and conditions are effective to hydrolyze the esters, which will be recognized by those of skill in the art.

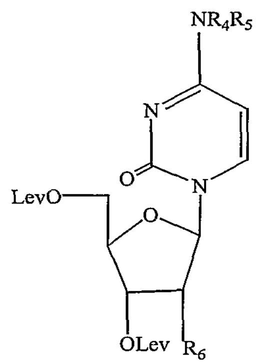
In some embodiments of the present invention, 3', 5'-di-*O*-levulinyl nucleosides are regioselectively deprotected at the 3'-*O*-levulinyl position by selecting a hydrolase, e.g. a lipase, effective to direct regioselective hydrolysis at the 3'-*O* -levulinyl position, without causing hydrolysis at the 5'-*O*-levulinyl position, and contacting the diprotected nucleoside with the hydrolase, (e.g. lipase) for a time and under conditions effective to hydrolyze the 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position. In some embodiments, the diprotected nucleosides have one of the following formulas:



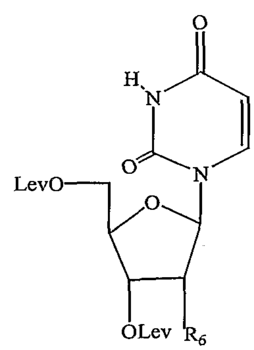
VI



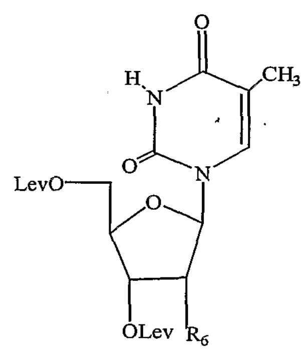
VII



VIII



IX



X

wherein:

R_6 is -H, or -OH;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

G is N or CH; and

Lev is -C(O)-$(CH_2)_2$-C(O)-CH_3.

For example, referring to Figure 2, 3'-*O*- selective hydrolysis was accomplished by reaction of 2 with immobilized *Pseudomonas cepacia* lipase [PSL-C, ratio of 1:3 w/w (2/PSL-C)] at 60 °C in 0.15M phosphate buffer giving the 5'-*O*-levulinyl derivative. *Candida antarctica* lipase A (CAL-A) also exhibited excellent selectivity towards the 3'-*O*-levulinyl position and has the advantage of requiring lower reaction temperatures than PSL-C (40 °C instead of 60 °C), shorter reaction times, and a lower ratio of enzyme/starting material (see Figure 4). Thus, 5'-*O*-levulinylthymidine (4a), *N*-benzoyl-5'-*O*-levulinyl-2'-deoxycytidine (4c), and *N*-benzoyl-5'-*O*-levulinyl-2'-deoxyadenosine (4e) were obtained with high yields (70-85%). The 3'-levulinyl regioisomer was not detected by TLC or ^{1}H-NMR of the crude reaction mixture. TLC showed traces of parent nucleosides 1.

N-isobutyryl-3',5'-di-*O*-levulinyl-2'-deoxyguanosine (2g) was not selectively hydrolyzed with CAL-A. However, treatment with PSL-C afforded the *N*-isobutyryl-5'-*O*-levulinyl-2'-deoxyguanosine (4g), which was isolated after 28 h at 60°C with 93% yield (entry 8, Table 1). *N*-Benzoyl-di-levulinyl derivatives (2c) and (2e) were both appropriate substrates for both lipases, PSL-C and CAL-A.

Treatment of 2'-OR nucleosides, as shown in Figure 3 with PSL-C or CAL-A yielded a mixture of 3'-O-levulinyl and 5'-O-levulinyl nucleosides, without the selectivity that was demonstrated with unprotected 2'-O and 2'-deoxynucleosides. While not being bound to any particular theory, this may be the result of steric hindrance caused by the 2'-O-R group, making the 3'-O-levulinyl group inaccessible for selective hydrolysis by either of these lipases.

In some embodiments of the present invention, enzymatic acylation may be carried out as depicted in Figure 5. A suspension of 1 (0.2 mmol), the oxime ester (0.6 mmol), and the lipase in dry THF (1 mL) under nitrogen was stirred at 250 rpm for the time and at the temperature indicated in Table 3 (Figure 8). The reactions were monitored by TLC (10% $MeOH/CH_2Cl_2$). The enzyme was filtered off and washed with CH_2Cl_2, the solvents were distilled under vacuum, and the residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and evaporated. The residue was precipitated in diethyl ether to afford after filtration the monolevulinyl nucleosides 3 or 4. No further purification was necessary except in entries 10 and 11 to separate the traces of other acyl derivatives.

As can be seen the acylation method requires fewer steps than the hydrolysis process, and is concomitantly more facile. The starting materials are the parent nucleosides, and is not necessary to first make the dilevulinyl derivatives. While the hydrolysis method requires two steps, the acylation method requires only one. The oxime ester is prepared in a single step and is purified by simple filtration. Flash chromatography can be avoided in the acylation process. By carrying out the acylation reaction in the presence of CAL-B, it is possible to obtain 5'-*O*-Lev-T, 5'-*O*-Lev-dC^{Bz}, 5'-*O*-Lev-dA^{Bz}, and 5'-*O*-Lev-dG^{iBu}. On the other hand, 3'-*O*-Lev-T, 3'-*O*-Lev-dC^{Bz}, and 3'-*O*-Lev-dA^{Bz} (but not 3'-*O*-Lev-dG^{iBu}) were obtained using PSL-C. Enzymatic hydrolysis reaction is necessary to prepare 3'-*O*-Lev-dG^{iBu}. Scale up of the reactions in Table 3 (Figure 6) resulted in increased yields, and flash chromatography

following enzyme filtration (i.e. avoiding extraction and precipitation) also results in higher yields.

In some embodiments of the present invention, the acylation method may be applied to ribonucleosides, such as those set forth in formulae XVI-XX. A general acylation method for acylating ribonucleosides is depicted in Figure 7, while data pertaining thereto are set forth in Table 4 (Figure 8). The monolevulinyl derivatives of 2'-alkylribonucleosides were soluble in ether and separation from the remaining oxime ester by precipitation is not possible. Thus, after enzyme filtration the residue was purified by flash chromatography (gradient eluent 5-20%MeOH/CH_2Cl_2).

Using the acylation method employing CAL-B, it is possible to obtain 5'-*O*-Lev-2'-OMOE-T, 5'-*O*-Lev-2'-*O*-MOE-5-Me-C, 5'-*O*-Lev-2'-*O*-MOE-5-Me-C^{Bz}, 5'-*O*-Lev-2'-*O*-MOE-A, 5'-*O*-Lev-2'-*O*-MOE-A^{Bz}, 5'-*O*-Lev-2'-*O*-Me-A, and 5'-*O*-Lev-2'-*O*-MOE-G^{iBu}. On the other hand, 3'-*O*-Lev-2'-*O*-MOE-5-Me-C^{Bz}, 3'-*O*-Lev-2'-*O*-MOE-A^{Bz}, and 3'-*O*-Lev-2'-*O*-Me-A^{Bz} were obtained using PSL-C. Hydrolysis reactions are required to prepare the corresponding 3'-Lev-T and 3'-Lev-G^{iBu} derivatives.

The nucleic acids useful in practicing the present invention include naturally occurring and non-naturally occurring nucleosides and nucleotides. The nucleosides and nucleotides of the present invention are not limited to monomer units but may also contain a plurality of linked monomer units, to form dinucleosides, nucleotides, and oligonucleotides and comprise naturally and non-naturally occurring nucleobases, sugars, and backbones.

Non-naturally occurring nucleosides and nucleotides may be modified by replacing the sugar moiety with an alternative structure which has primary and secondary alcohol groups similar to those of ribose. Non-naturally occurring sugars and nucleosidic bases are typically structurally distinguishable from, yet functionally interchangeable with, naturally occurring sugars (e.g. ribose and deoxyribose) and nucleosidic bases (e.g., adenine, guanine, cytosine, thymine). Thus, non-naturally occurring nucleobases and sugars include all such structures which mimic the structure and/or function of naturally occurring species, and which aid in the binding of the oligonucleotide to a target, or which otherwise advantageously contribute to the properties of the oligonucleotide.

Backbone modifications include modifications to the phosphate backbone to increase the resistance to nucleases. These modifications include use of linkages such as methyl phosphonates, phosphorothioates and phosphorodithioates as well as those modifications that

dramatically alter the nature of the internucleotide linkage such as non-phosphorus linkages, peptide nucleic acids (PNAs) and 2'-5' linkages.

A heterocyclic base moiety (often referred to in the art simply as a "base" or a "nucleobase") amenable to the present invention includes both naturally and non-naturally occurring nucleobases. The heterocyclic base moiety further may be protected wherein one or more functionalities of the base bears a protecting group. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine and guanine, and the pyrimidine bases thymine, cytosine and uracil. Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie, International Edition*, 1991, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993.

In some embodiments according to the present invention, the processes of the present invention are applicable to so-called C-nucleosides (i.e. nucleosides in which the nucleobase is linked to the nucleoside ring via a C-C bond, as opposed to the naturally-occurring C-N bond). Also, in some embodiments of the present invention, the nucleoside ring is in the α-configuration (as opposed to the naturally-occurring β-configuration), the L-configuration (as opposed to the naturally-occurring D-configuration) or both (i.e. the α-L-configuration).

Certain heterocyclic base moieties are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention to complementary targets. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine

substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C (*Id.*, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with selected 2'-sugar modifications such as 2'-methoxyethyl groups.

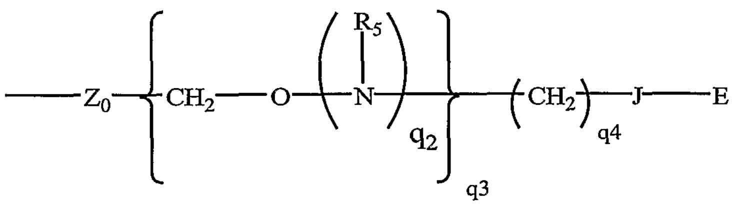
Representative United States patents that teach the preparation of heterocyclic base moieties (modified nucleobases) include, but are not limited to, U.S. Patents 3,687,808; 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/762,587, filed on December 10, 1996, also herein incorporated by reference.

A representative list of 2'-substituent groups amenable to the present invention include C_1-C_{20} alkyl, C_2-C_{20} alkenyl, C_2-C_{20} alkynyl, C_5-C_{20} aryl, O-alkyl, O-alkenyl, O-alkynyl, O-alkylamino, O-alkylalkoxy, O-alkylaminoalkyl, O-alkyl imidazole, S-alkenyl, S-alkynyl, NH-alkyl, NH-alkenyl, NH-alkynyl, N-dialkyl, O-aryl, S-aryl, NH-aryl, O-aralkyl, S-aralkyl, NH-aralkyl, N-phthalimido, halogen (particularly fluoro), keto, carboxyl, nitro, nitroso, nitrile, trifluoromethyl, trifluoromethoxy, imidazole, azido, hydrazino, hydroxylamino, isocyanato, sulfoxide, sulfone, sulfide, disulfide, silyl, heterocycle, carbocycle, polyamine, polyamide, polyalkylene glycol, and polyethers of the formula $(O\text{-alkyl})_m$, where m is 1 to about 10. Preferred among these polyethers are linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and those which are disclosed by Ouchi *et al.* (*Drug Design and Discovery* 1992, *9*, 93), Ravasio *et al.* (*J. Org. Chem.* 1991, *56*, 4329) and Delgardo *et. al.* (*Critical Reviews in Therapeutic Drug Carrier Systems* 1992, *9*, 249), each of which is herein incorporated by reference in its entirety. Further sugar modifications are disclosed in Cook, P.D., *Anti-Cancer Drug Design*, 1991, *6*, 585-607. Fluoro, O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl amino substitution is described in United States Patent Application serial number 08/398,901, filed March 6, 1995, entitled Oligomeric Compounds having Pyrimidine Nucleotide(s) with 2' and 5' Substitutions, hereby incorporated by reference in its entirety.

Additional substituent groups amenable to the present invention include -SR and $-NR_2$ groups, wherein each R is, independently, hydrogen, a protecting group or substituted or unsubstituted alkyl, alkenyl, or alkynyl. 2'-SR nucleosides are disclosed in United States Patent No. 5,670,633, issued September 23, 1997, hereby incorporated by reference in its

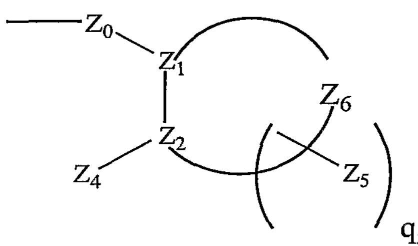
entirety. The incorporation of 2'-SR monomer synthons are disclosed by Hamm *et al., J. Org. Chem.,* 1997, *62,* 3415-3420. 2'-NR_2 nucleosides are disclosed by Goettingen, M., *J. Org. Chem.,* 1996, *61*, 73-6281; and Polushin *et al., Tetrahedron Lett.,* 1996, *37*, 3227-3230.

Further substituent groups have one of formulae XXI or XXII:

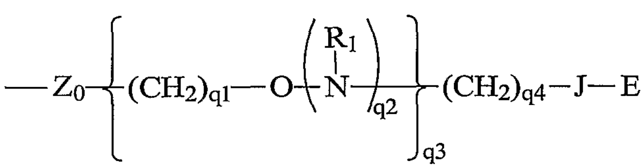


(XXI)

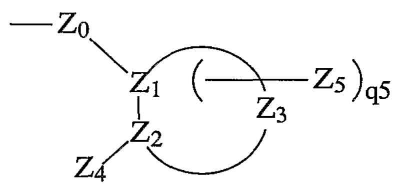
or



(XXII)



I



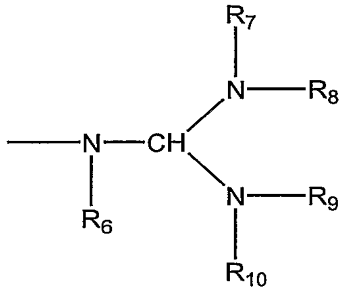
II

wherein:

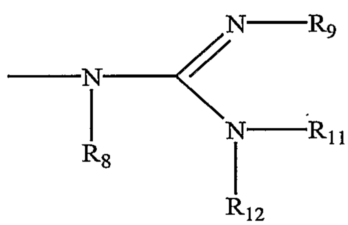
Z_0 is O, S or NH;

J is a single bond, O or C(=O);

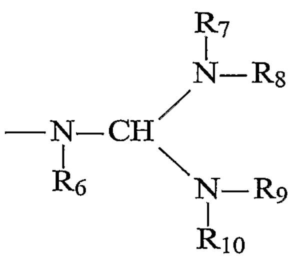
E is C_1-C_{10} alkyl, $N(R_1)(R_2)$, $N(R_1)(R_5)$, $N=C(R_1)(R_2)$, $N=C(R_1)(R_5)$ or has one of formula XXIII or XXIV;



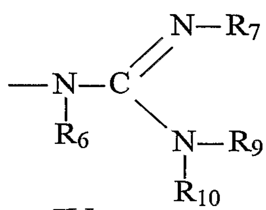
(XXIII)



(XXIV)



III



IV

each R_6, R_7, R_8, R_9 and R_{10} is, independently, hydrogen, $C(O)R_{11}$, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional group or a conjugate group, wherein the substituent groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

or optionally, R_7 and R_8, together form a phthalimido moiety with the nitrogen atom to which they are attached;

or optionally, R_9 and R_{10}, together form a phthalimido moiety with the nitrogen atom to which they are attached;

each R_{11} and R_{12} is, independently, substituted or unsubstituted C_1-C_{10} alkyl, trifluoromethyl, cyanoethyloxy, methoxy, ethoxy, t-butoxy, allyloxy, 9-fluorenylmethoxy, 2-(trimethylsilyl)-ethoxy, 2,2,2-trichloroethoxy, benzyloxy, butyryl, iso-butyryl, phenyl or aryl;

R_5 is T-L,

T is a bond or a linking moiety;

L is a chemical functional group, a conjugate group or a solid support material;

each R_1 and R_2 is, independently, H, a nitrogen protecting group, substituted or unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl, substituted or unsubstituted C_2-C_{10} alkynyl, wherein said substitution is OR_3, SR_3, NH_3^+, $N(R_3)(R_4)$, guanidino or acyl where said acyl is an acid amide or an ester;

or R_1 and R_2, together, are a nitrogen protecting group or are joined in a ring structure that optionally includes an additional heteroatom selected from N and O;

or R_1, T and L, together, are a chemical functional group;

each R_3 and R_4 is, independently, H, C_1-C_{10} alkyl, a nitrogen protecting group, or R_3 and R_4, together, are a nitrogen protecting group;

or R_3 and R_4 are joined in a ring structure that optionally includes an additional heteroatom selected from N and O;

Z_4 is OX, SX, or $N(X)_2$;

each X is, independently, H, C_1-C_8 alkyl, C_1-C_8 haloalkyl, $C(=NH)N(H)R_5$, $C(=O)N(H)R_5$ or $OC(=O)N(H)R_5$;

R_5 is H or C_1-C_8 alkyl;

Z_1, Z_2 and Z_3 comprise a ring system having from about 4 to about 7 carbon atoms or having from about 3 to about 6 carbon atoms and 1 or 2 hetero atoms wherein said hetero atoms are selected from oxygen, nitrogen and sulfur and wherein said ring system is aliphatic, unsaturated aliphatic, aromatic, or saturated or unsaturated heterocyclic;

Z_5 is alkyl or haloalkyl having 1 to about 10 carbon atoms, alkenyl having 2 to about 10 carbon atoms, alkynyl having 2 to about 10 carbon atoms, aryl having 6 to about 14 carbon atoms, $N(R_1)(R_2)$ OR_1, halo, SR_1 or CN;

each q_1 is, independently, an integer from 1 to 10;

each q_2 is, independently, 0 or 1;

q_3 is 0 or an integer from 1 to 10;

q_4 is an integer from 1 to 10;

q_5 is from 0, 1 or 2; and

provided that when q_3 is 0, q_4 is greater than 1.

Representative substituent groups of Formula I are disclosed in United States Patent Application Serial No. 09/130,973, filed August 7, 1998, entitled “Capped 2'-Oxyethoxy Oligonucleotides,” hereby incorporated by reference in its entirety.

Representative cyclic substituent groups of Formula II are disclosed in United States Patent Application Serial No. 09/123,108, filed July 27, 1998, entitled “RNA Targeted 2'-Modified Oligonucleotides that are Conformationally Preorganized,” hereby incorporated by reference in its entirety.

Particularly preferred substituent groups include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, $O(CH_2)_nON[(CH_2)_nCH_3)]_2$ (where n and m are from 1 to about 10), C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino and substituted silyl. Another particularly preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$ or 2'-MOE, Martin *et al.*, *Helv. Chim. Acta*, 1995, *78*, 486). A further preferred substituent group is 2’-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE. Representative aminooxy substituent groups are described in co-owned United States Patent Application serial number 09/344,260, filed June 25, 1999, entitled "Aminooxy-Functionalized Oligomers"; and United States Patent Application serial number 09/370,541, filed August 9, 1999, also identified by attorney docket number ISIS-3993, entitled Aminooxy-Functionalized Oligomers and Methods for Making Same; hereby incorporated by reference in their entirety.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on nucleosides and oligomers, particularly the 3' position of the sugar on the 3' terminal nucleoside or at a 3'-position of a nucleoside that has a linkage from the 2'-position such as a 2’-5’ linked oligomer and at the 5'-position at a 5'-terminus. Oligomers may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned, and each of which is herein incorporated by

reference, and commonly owned United States patent 5,859,221, also herein incorporated by reference.

Representative guanidino substituent groups that are shown in formula III and IV are disclosed in co-owned United States Patent Application 09/349,040, entitled "Functionalized Oligomers", filed July 7, 1999, hereby incorporated by reference in its entirety.

Representative acetamido substituent groups are disclosed in United States Patent Application 09/378,568, entitled "2'-O-Acetamido Modified Monomers and Oligomers", filed August 19, 1999, also identified by attorney docket number ISIS-4071, hereby incorporated by reference in its entirety.

Representative dimethylaminoethyloxyethyl substituent groups are disclosed in International Patent Application PCT/US99/17895, entitled "2'-O-Dimethylaminoethyloxy-ethyl-Modified Oligonucleotides", filed August 6, 1999, also identified by attorney docket number ISIS-4045, hereby incorporated by reference in its entirety.

The methods of the present invention use labile protecting groups to protect various functional moieties during synthesis. Protecting groups are used ubiquitously in standard oligonucleotide synthetic regimes for protection of several different types of functionality. In general, protecting groups render chemical functionality inert to specific reaction conditions and can be appended to and removed from such functionality in a molecule without substantially damaging the remainder of the molecule. *See, e.g.*, Green and Wuts, Protective Groups in Organic Synthesis, 2d edition, John Wiley & Sons, New York, 1991. Representative protecting groups useful to protect nucleotides during synthesis include base labile protecting groups and acid labile protecting groups. Base labile protecting groups are used to protect the exocyclic amino groups of the heterocyclic nucleobases. This type of protection is generally achieved by acylation. Two commonly used acylating groups for this purpose are benzoyl chloride and isobutyryl chloride. These protecting groups are stable to the reaction conditions used during oligonucleotide synthesis and are cleaved at approximately equal rates during the base treatment at the end of synthesis.

Hydroxyl protecting groups typically used in oligonucleotide synthesis may be represented by the group having the formula: $-C(R_1)(R_2)(R_3)$ wherein each of R_1, R_2 and R_3 is an unsubstituted or mono-substituted aryl or heteroaryl group selected from phenyl, naphthyl, anthracyl, and five or six membered heterocylic rings with a single heteroatom selected from N, O and S, or two N heteroatoms, including quinolyl, furyl, and thienyl; where the substituent

is selected from halo (i.e., F, Cl, Br, and I), nitro, C_1-C_4-alkyl or alkoxy, and aryl, aralkyl and cycloalkyl containing up to 10 carbon atoms; and wherein R_2 and R_3 may each also be C_1-C_4-alkyl or aralkyl or cycloalkyl containing up to 10 carbon atoms.

In the present context, an acylating agent for introducing the levulinyl group is an acylating agent that is capable of reacting with an active group, such as hydroxyl, to produce an acylated group, wherein the acyl portion is the levulinyl group as defined above. Such acylating reagents include levulinic acid, as well as activated forms of levulinic acid, including the anhydride, esters, oximes and halic acid derivatives thereof. Levulinic acid is a preferred acylating agent for introducing the levulinyl group.

The present invention contemplates the use of a hydrolase, such as a lipase, to affect either deprotection or acylation of a nucleoside or modified nucleoside. Several groups of hydrolases exist in nature. Two of these groups of hydrolases have been widely used in non-natural media: lipases and proteases. From a chemical point-of-view, lipases and proteases in organic solvents can be seen as mild and selective reagents that are able to activate a generic carboxylate and transfer it to a large number of nucleophiles. The present invention contemplates the use of such hydrolases in the acylation (to introduce a levulinyl group onto a nucleoside) or deprotection (i.e. removal of a levulinyl group from a protected nucleoside). The term hydrolase thus includes lipases, proteases, and similar hydrolases capable of regioselectively directing acylation of a nucleoside or deprotection of an acylated nucleoside.

As will be recognized, additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon examination of the following examples thereof, which are not intended to be limiting.

EXPERIMENTAL

General. *Candida antarctica* lipase B (CAL-B) was a gift from Novo Nordisk Co. *Candida antarctica* lipase A (CAL-A) and immobilized *Pseudomonas cepacia* lipase (PSL-C) were purchased from Roche Diagnostics S. L. and Amano Pharmaceuticals, respectively. PS-carbodiimide was purchased from Argonaut Technologies (San Carlos, CA, EE.UU.). All other reagents were purchased from Aldrich or Fluka. Solvents were distilled over an adequate desiccant under nitrogen.

3',5'-di-*O*-Levulinyl-2'-deoxynucleosides (2).

Method A: To a stirred mixture of 1 (2 mmol) and Et_3N (1.7 mL, 12 mmol) in 1,4-dioxane (20 mL) under nitrogen, was added levulinic acid (1.21 g, 10.4 mmol), DCC (2.14 g, 10.4 mmol), and DMAP (20 mg, 0.16 mmol). The reaction was stirred at room temperature for 3 hours. In order to minimize the formation of triprotected cytidine derivative, 6 mmol both of LevOH and DCC, and 5 mmol of Et_3N were used for 1b. The insoluble material was collected by filtration and the filtrate was evaporated under vacuum. The residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4 and evaporated. Cold Et_2O was added, and the slurry was scratched until crystallization occurs. The solid was filtered and washed with cold Et_2O, and then was poured in EtOAc (MeOH in case of 2f). The insoluble material was filtered and the filtrate was concentrated to afford the title compounds. The resulting materials were pure enough to be carried directly on to the enzymatic hydrolysis step. Further purification by flash chromatography (EtOAc) give pure 3',5'-di-*O*-levulinylnucleosides 2a-g.

Method B: To a stirred mixture of 1 (0.4 mmol) and Et_3N (0.15 mL, 1 mmol) in 1,4-dioxane (5 mL) under nitrogen, was added levulinic acid (0.14 g, 1.2 mmol), PS-carbodiimide (1.05 g, 1.2 mmol), DMAP (4 mg, 0.032 mmol), and DMAP•HCl (3 mg, 0.02 mmol). The reaction was stirred at room temperature for 3 hours. The insoluble material was collected by filtration and the filtrate was evaporated under vacuum. The residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4 and evaporated. The solid was washed with cold Et_2O to afford 3',5'-di-*O*-levulinylnucleosides 2a and 2d.

3',5'-di-*O*-Levulinylthymidine (2a). $\underline{R_f}$ (10% MeOH/CH_2Cl_2): 0.45; Mp: 87-89 °C; IR (KBr): υ 3315, 3074, 3006, 2967, 2947, 1743, 1689, and 1660 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): d 1.88 (s, 3H, Me), 2.14 (s, 3H, *Me*-Lev), 2.15 (s, 3H, *Me*-Lev), 2.18 (m, 1H, $H_{2'}$), 2.41 (m, 1H, $H_{2'}$), 2.54 (m, 4H, 2CH_2-Lev), 2.73 (m, 4H, 2CH_2-Lev), 4.19 (m, 1H, $H_{4'}$), 4.31 (m, 2H, $H_{5'}$), 5.18 (m, 1H, H_3), 6.28 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 8.5, $^3J_{HH}$ 5.4 Hz), 7.32 (s,1H, H_6), and 9.99 (s,1H,N*H*); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): d 12.3 (Me), 27.48 (*C*H_2-Lev), 27.54 (*C*H_2-Lev), 29.4 (2 Me-Lev), 36.8 ($C_{2'}$), 37.5 (2*C*H_2-Lev), 63.7 ($C_{5'}$), 74.2, 81.8, 84.3 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 111.2 (C_5), 134.6 (C_6), 150.3 (C_2), 163.8 (C_4), 171.97 (*C*=O Lev), 172.02 (*C*=O Lev), and 206.3 (2*C*=O Lev); MS (ESI$^+$, *m/z*): 439 [$(M+H)^+$, 100%], and 461 [$(M+Na)^+$, 50].

3',5'-di-*O*-Levulinyl-2'-deoxycytidine (2b). R$_f$ (20% MeOH/CH_2Cl_2): 0.67; IR (KBr): υ 3390, 2940, 1737, 1715, and 1649 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 2.39 (s, 3H, Me-Lev), 2.40 (s, 3H, Me-Lev), 2.43 (m, 1H, $H_{2'}$), 2.75 (m, 1H, $H_{2'}$), 2.79 (m, 4H, 2CH_2-Lev), 3.02 (m, 4H, 2CH_2-Lev), 4.51 (m, 3H, $H_{4'}$+2$H_{5'}$), 5.46 (m, 1H, $H_{3'}$), 6.17 (d, 1H, H_5, $^3J_{HH}$ 7.6 Hz), 6.45 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 8.6, $^3J_{HH}$ 5.7 Hz), and 7.98 (dd, 1H, H_6, $^3J_{HH}$ 7.3 Hz); ^{13}C-NMR (MeOH-d$_4$, 75.5 MHz): d 29.1 (*C*H$_2$-Lev), 29.2 (*C*H$_2$-Lev), 29.9 (*Me*-Lev), 38.9 (2*C*H$_2$-Lev), 39.2 ($C_{2'}$), 65.5 ($C_{5'}$), 76.5, 84.1, 87.9 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 96.8 (C_5), 142.2 (C_6), 158.4 (C_2), 168.0 (C_4), 174.2 (*C*=O), 174.4 (*C*=O), and 209.7 (*C*=O); MS (ESI$^+$, *m/z*): 446 [(M+Na)$^+$, 70%] and 462 [(M+K)$^+$, 100].

N-Benzoyl-3',5'-di-*O*-levulinyl-2'-deoxycytidine (2c). R$_f$ (10% MeOH/CH_2Cl_2): 0.61; Mp: 107-109 °C; IR (KBr): υ 3233, 1744, 1731, and 1668 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 2.31 (s, 3H, *Me*-Lev), 2.38 (s, 3H, *Me*-Lev), 2.50 (m, 1H, $H_{2'}$), 2.75 (m, 4H, 2CH_2-Lev), 2.95 (m, 1H, $H_{2'}$), 3.05 (m, 4H, 2CH_2-Lev), 4.59 (m, 3H, $H_{4'}$+2$H_{5'}$), 5.49 (m, 1H, $H_{3'}$), 6.42 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 7.7, $^3J_{HH}$ 5.7 Hz), 7.75 (m, 4H, H_5+H_m+H_p), 8.15 (m, 2H, H_o), and 8.45 (d, 1H, H_6, $^3J_{HH}$ 7.6 Hz); ^{13}C-NMR (MeOH-d$_4$, 75.5 MHz): d 29.1 (*C*H$_2$-Lev), 29.2 (*C*H$_2$-Lev), 29.9 (*Me*-Lev), 38.91 (*C*H$_2$-Lev), 38.94 (*C*H$_2$-Lev), 39.8 ($C_{2'}$), 65.3 ($C_{5'}$), 76.5, 85.0, 89.3 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 99.0 (C_5), 129.5, 130.1 (C_o+C_m), 134.4 (C_p), 135.0 (C_i), 146.2 (C_6), 158.0 (C_2), 165.1 (C_4), 169.2 (Ph*C*=O), 174.3 (*C*=O), 174.4 (*C*=O), 209.67 (*C*=O), and 209.72 (*C*=O); MS (ESI$^+$, *m/z*): 528 [(M+H)$^+$, 100%], 550 [(M+Na)$^+$, 30], and 566 [(M+K)$^+$, 40].

3',5'-di-*O*-Levulinyl-2'-deoxyadenosine (2d). R$_f$ (10% MeOH/CH_2Cl_2): 0.44; IR (KBr): υ 3418, 3165, 2923, 1738, 1715, and 1644 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 2.33 (s, 3H, *Me*-Lev), 2.39 (s, 3H, *Me*-Lev), 2.79 (m, 5H, 2CH_2-Lev+1$H_{2'}$), 3.00 (m, 4H, 2CH_2-Lev), 3.25 (m, 1H, $H_{2'}$), 4.52 (m, 3H, $H_{4'}$+2$H_{5'}$), 5.65 (m, 1H, $H_{3'}$), 6.61 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 6.0, $^3J_{HH}$ 7.9 Hz), 8.41 (s, 1H, H_2 or H_8), and 8.50 (s, 1H, H_8 or H_2); ^{13}C-NMR (MeOH-d$_4$, 75.5 MHz): d 29.1 (*C*H$_2$-Lev), 29.2 (*C*H$_2$-Lev), 29.9 (2*Me*-Lev), 38.1 ($C_{2'}$), 38.9 (2*C*H$_2$-Lev), 65.2 ($C_{5'}$), 76.5, 84.2, 86.2 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 120.8 (C_5), 141.2 (C_8), 150.7 (C_4), 154.2 (C_2), 157.6 (C_6), 174.2 (*C*=O), 174.4 (*C*=O), 209.68 (*C*=O), and 209.73 (*C*=O); MS (ESI$^+$, *m/z*): 448 [(M+H)$^+$, 20%], 470 [(M+Na)$^+$, 80], and 486 [(M+K)$^+$, 100].

N-Benzoyl-3',5'-di-*O*-levulinyl-2'-deoxyadenosine (2e). R$_f$ (10% MeOH/CH_2Cl_2): 0.71; Mp: 69-71 °C; IR (KBr): υ 3412, 3086, 2958, 1738, 1714, and 1685 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 300 MHz): d 2.28 (s, 3H, *Me*-Lev), 2.35 (s, 3H, *Me*-Lev), 2.75 (m, 4H, 2CH_2-

Lev), 2.87 (m, 1H, $H_{2'}$), 2.99 (m, 4H, 2CH_2-Lev), 3.30 (m, 1H, $H_{2'}$), 4.52 (m, 3H, $H_{4'}$+2$H_{5'}$), 5.65 (m, 1H, $H_{3'}$), 6.70 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 6.8 Hz), 7.75 (m, 3H, 2H_m+H_p), 8.25 (apparent d, 2H, 2H_o, $^3J_{HH}$ 7.4 Hz), 8.75 (s, 1H, H_2 or H_8), and 8.88 (s, 1H, H_8 or H_2); ^{13}C-NMR (MeOH-d_4, 75.5 MHz): d 29.0 (CH$_2$-Lev), 29.2 (CH$_2$-Lev), 30.0 (*Me*-Lev), 37.9 ($C_{2'}$), 38.87 (CH$_2$-Lev), 38.91 (CH$_2$-Lev), 65.2 ($C_{5'}$), 76.4, 84.3, 86.5 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 125.5 (C_5), 129.7, 130.0 (C_o+C_m), 134.1 (C_p), 135.1 (C_i), 144.5 (C_8), 151.3 (C_4), 153.3(C_6), 153.5 (C_2), 168.1 (PhC=O), 174.2 (C=O), 174.3 (C=O), 209.6 (C=O), and 209.7 (C=O); MS (ESI$^+$, *m/z*): 552 [(M+H)$^+$, 100%] and 574 [(M+Na)$^+$, 17].

3',5'-di-*O*-Levulinyl-2'-deoxyguanosine (2f). R_f (20% MeOH/CH_2Cl_2): 0.65; Mp: 148-150 °C; IR (KBr): υ 3397, 3153, 2940, and 1711 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 200 MHz): d 2.16 (s, 3H, *Me*-Lev), 2.22 (s, 3H, *Me*-Lev), 2.60 (m, 5H, 2CH_2-Lev+1$H_{2'}$), 2.83 (m, 4H, 2CH_2-Lev), 3.00 (m, 1H, $H_{2'}$), 4.29 (m, 3H, $H_{4'}$+2$H_{5'}$), 5.35 (m, 1H, $H_{3'}$), 6.22 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 5.8, $^3J_{HH}$ 8.8 Hz), 6.69 (br s, 2H, NH), and 8.00 (s, 1H, H_8); ^{13}C-NMR (DMSO-d_6, 50.3 MHz): d 27.5 (CH$_2$-Lev), 27.7(CH$_2$-Lev), 29.55 (*Me*-Lev), 29.60 (*Me*-Lev), 35.5, 37.4, 37.50 (2CH$_2$-Lev+$C_{2'}$), 63.8 ($C_{5'}$), 74.7, 81.5, 82.6 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 116.8 (C_5), 135.1 (C_8), 151.2 (C_4), 154.0 (C_2), 156.9 (C_6), 172.1 (C=O), 172.2 (C=O), 206.9 (C=O), and 207.1 (C=O); MS (ESI$^+$, *m/z*): 464 [(M+H)$^+$, 22%], 486 [(M+Na)$^+$, 75], and 502 [(M+K)$^+$, 100].

N-Isobutyryl-3',5'-di-*O*-levulinyl-2'-deoxyguanosine (2g). R_f (20% MeOH/CH_2Cl_2): 0.85; Mp: 45-47 °C; IR (KBr): υ 3413, 2935, 1740, 1714, 1680, and 1613 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 200 MHz): d 1.23 (d, 6H, *Me*-^{i}Bu, $^3J_{HH}$ 6.5 Hz), 2.15 (s, 3H, *Me*-Lev), 2.20 (s, 3H, *Me*-Lev), 2.55-3.19 (several m, 11H, 4CH_2-Lev+2$H_{2'}$+CH-^{i}Bu), 4.32 (m, 3H, $H_{4'}$+2$H_{5'}$), 5.35 (m, 1H, $H_{3'}$), 6.35 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 7.2 Hz), 8.35 (s, 1H, H_8), 11.80 (br s, 1H, NH), and 12.20 (br s, 1H, NH); ^{13}C-NMR (DMSO-d_6, 50.3 MHz): d 18.86 (*Me*-^{i}Bu), 18.91 (*Me*-^{i}Bu), 27.5 (CH$_2$-Lev), 27.6 (CH$_2$-Lev), 29.5 (*Me*-Lev), 29.6 (*Me*-Lev), 34.8 (CH-^{i}Bu), 35.5 ($C_{2'}$), 37.38 (CH$_2$-Lev), 37.45 (CH$_2$-Lev), 63.7 ($C_{5'}$), 74.6, 81.7, 82.9 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 120.3 (C_5), 137.3 (C_8), 148.3, 148.7 (C_2+C_4), 154.8 (C_6), 172.1 (C=O), 172.2 (C=O), 180.2 (^{i}Bu-C=O), 206.9 (C=O), and 207.1 (C=O); MS (ESI$^+$, *m/z*): 534 [(M+H)$^+$, 100%], 556 [(M+Na)$^+$, 60], and 572 [(M+K)$^+$, 27].

General procedure for the enzymatic hydrolysis of 3',5'-di-*O*-levulinyl-2'-deoxynucleosides. To a solution of 2 (0.2 mmol) in 1,4-dioxane (0.35 mL) was added 0.15M phosphate buffer pH= 7 (1.65 mL) and the corresponding lipase [ratio of 2:enzyme was 1:1

(w/w) for CAL-A or CAL-B, and 1:3 (w/w) for PSL-C]. The mixture was allowed to react at 250 rpm for the time and at the temperature indicated in Table 1. The reactions were monitored by TLC (10% MeOH/CH_2Cl_2). The enzyme was filtered off and washed with CH_2Cl_2, the solvents were distilled under vacuum, and the residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and evaporated to give monoacylnucleosides 3 or 4. In case of 3b, the residue was purified by flash chromatography instead of extraction.

3'-*O*-Levulinylthymidine (3a). R_f (10% MeOH/CH_2Cl_2): 0.32; Mp: 50-52 °C; IR (KBr): υ 3449, 3065, 2927, and 1706 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 2.09 (d, 3H, Me, J_{HH} 1.3 Hz), 2.39 (s, 3H, *Me*-Lev), 2.57 (m, 2H, $H_{2'}$), 2.80 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.0 Hz), 3.05 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.2 Hz), 4.01 (m, 2H, $H_{5'}$), 4.29 (m, 1H, $H_{4'}$), 5.02 (m, 1H, $H_{3'}$), 6.50 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 8.1, $^3J_{HH}$ 6.5 Hz), and 8.04 (d, 1H, H_6, J_{HH} 1.3 Hz); ^{13}C-NMR ($CDCl_3$, 75.5 MHz): d 12.4 (Me), 27.8 (CH_2-Lev), 29.6 (*Me*-Lev), 37.1 ($C_{2'}$), 37.7 (CH_2-Lev), 62.2 ($C_{5'}$), 74.9, 85.0, 85.7 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 111.1 (C_5), 136.5 (C_6), 150.6 (C_2), 164.3 (C_4), 172.4 (2*C*=O Lev), and 206.8 (2*C*=O Lev); MS (ESI^+, *m/z*): 363 [$(M+Na)^+$, 100%] and 379 [$(M+K)^+$, 30].

3'-*O*-Levulinyl-2'-deoxycytidine (3b). R_f (20% MeOH/CH_2Cl_2): 0.41; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 2.39 (s, 3H, *Me*-Lev), 2.47 (m, 1H, $H_{2'}$), 2.67 (m, 1H, $H_{2'}$), 2.75 (m, 2H, CH_2-Lev), 3.02 (m, 2H, CH_2-Lev), 4.00 (m, 2H, 2$H_{5'}$), 4.30 (m, 1H, $H_{4'}$), 5.49 (m, 1H, $H_{3'}$), 6.15 (d, 1H, H_5, $^3J_{HH}$ 6.8 Hz), 6.48 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 6.8 Hz), and 8.32 (d, 1H, H_6, $^3J_{HH}$ 7.3 Hz); ^{13}C-NMR (MeOH-d_4, 75.5 MHz): d 29.2 (CH_2-Lev), 29.9 (*Me*-Lev), 38.9, 39.5 ($C_{2'}$+CH_2-Lev), 63.2 ($C_{5'}$), 76.9, 87.1,87.8 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 96.6 (C_5), 143.0 (C_6), 158.2 (C_2), 167.6 (C_4), 174.2 (*C=O*), and 209.8 (*C*=O).

3'-*O*-Levulinyl-2'-deoxyadenosine (3d). R_f (20% MeOH/CH_2Cl_2): 0.66; IR (KBr): υ 3292, 2925, 1730, 1715, 1690, 1644, and 1610 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 2.40 (s, 3H, *Me*-Lev), 2.76 (m, 1H, $H_{2'}$), 2.80 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.2 Hz), 3.05 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.2 Hz), 3.14 (m, 1H, $H_{2'}$), 4.04 (m, 2H, 2$H_{5'}$), 4.40 (m, 1H, $H_{4'}$), 5.66 (d, 1H, $H_{3'}$, $^3J_{HH}$ 6.0 Hz), 6.61(dd, 1H, $H_{1'}$, $^3J_{HH}$ 5.7, $^3J_{HH}$ 9.1 Hz), 8.39 (s, 1H, H_2 or H_8), and 8.50 (s, 1H, H_8 or H_2); ^{13}C-NMR (MeOH-d_4, 75.5 MHz): d 29.1 (CH_2-Lev), 29.9 (*Me*-Lev), 38.9, 39.0 ($C_{2'}$+CH_2-Lev), 64.0 ($C_{5'}$), 77.5, 87.6, 87.9 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 121.2 (C_5), 141.9 (C_8), 150.1 (C_4),

153.8 (C_2), 157.8 (C_6), 174.3 (C=O), and 209.8 (C=O); MS (ESI$^+$, m/z): 350 [(M+H)$^+$, 100%], 372 [(M+Na)$^+$, 100], and 388 [(M+K)$^+$, 60].

N-Isobutyryl-3'-*O*-levulinyl-2'-deoxyguanosine (3g). R_f (20% MeOH/CH_2Cl_2): 0.75; Mp: 170-172 °C; IR (KBr): υ 3415, 2961, 2929, 2859, 1725, 1686, and 1614 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 1.41 (d, 6H, *Me*-^{i}Bu, $^3J_{HH}$ 6.8 Hz), 2.38 (s, 3H, *Me*-Lev), 2.70-3.09 (m, 7H, 2CH_2-Lev+2$H_{2'}$+C*H*-^{i}Bu), 3.98 (d, 2H, 2$H_{5'}$, $^3J_{HH}$ 3.4 Hz), 4.45 (m, 1H, $H_{4'}$), 5.60 (m, 1H, $H_{3'}$), 6.51 (dd, 1H, $H_{1'}$, $^3J_{HH}$ 5.9, $^3J_{HH}$ 8.4 Hz), and 8.45 (s, 1H, H_8); ^{13}C-NMR (MeOH-d_4, 50.3 MHz): d 19.6 (*Me*-^{i}Bu), 29.2 (CH$_2$-Lev), 30.0 (*Me*-Lev), 37.2 (CH-^{i}Bu), 38.9, 39.3 ($C_{2'}$+CH$_2$-Lev), 63.3 ($C_{5'}$), 76.8, 85.8, 87.3 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 121.5 (C_5), 139.7 (C_8), 150.0, 150.5 (C_2+C_4), 157.6 (C_6), 174.2 (C=O), 182.0 (^{i}Bu-C=O), and 209.7 (C=O); MS (ESI$^+$, m/z): 436 [(M+H)$^+$, 15%] and 458 [(M+Na)$^+$, 50].

5'-*O*-Levulinylthymidine (4a). R_f (10% MeOH/CH_2Cl_2): 0.22; Mp: 141-143 °C; IR (KBr): υ 3393, 3215, 2934, 1737, 1724, 1643, and 1629 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 200 MHz): d 1.91 (s, 3H, Me), 2.27 (s, 3H, *Me*-Lev), 2.30 (m, 2H, $H_{2'}$), 2.66 (m, 2H, CH_2-Lev), 2.89 (t, 2H, CH_2-Lev, $^3J_{HH}$ 6.2 Hz), 4.07 (m, 1H, $H_{4'}$), 4.35 (m, 3H, $H_{3'}$+2$H_{5'}$), 5.55 (d, 1H, O*H*), 6.32 (t, 1H, $H_{1'}$, $^3J_{HH}$ 7.0 Hz), 7.6 (s, 1H, $H_{6'}$), and 11.45 (s,1H,N*H*); ^{13}C-NMR (DMSO-d_6, 50.3 MHz): d 12.02 (Me), 27.4 (CH$_2$-Lev), 29.4 (*Me*-Lev), 37.2 ($C_{2'}$), 38.4 (CH$_2$-Lev), 63.8 ($C_{5'}$), 70.1 ($C_{3'}$), 83.5, 83.6 ($C_{1'}$+$C_{4'}$), 109.7 (C_5), 135.7 (C_6), 150.3 (C_4), 163.6 (C_2), 172.1 (C=O Lev), and 206.7 (C=O Lev); MS (ESI$^+$, m/z): 341 [(M+H)$^+$, 40%], 379 [(M+Na)$^+$, 100], and 379 [(M+K)$^+$, 80].

N-Benzoyl-5'-*O*-levulinyl-2'-deoxycytidine (4c). R_f (10% MeOH/CH_2Cl_2): 0.37; Mp: 50-52 °C. IR (KBr): υ 3410, 2919, 1738, 1701, and 1650 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 300 MHz): d 2.20 (s, 3H, *Me*-Lev), 2.25 (m, 1H, $H_{2'}$), 2.58 (m, 2H, CH_2-Lev), 2.75 (m, 1H, $H_{2'}$), 2.82 (m, 2H, CH_2-Lev), 3.35 (s, 1H, O*H*), 4.25 (m, 1H, $H_{3'}$), 4.40 (m, 3H, 2$H_{5'}$+$H_{4'}$), 6.30 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 6.2 Hz), 7.55 (m, 4H, H_5+2H_m+H_p), 7.90 (apparent d, 2H, H_o, $^3J_{HH}$ 7.1 Hz), 8.20 (d, 1H, H_6, $^3J_{HH}$ 7.4 Hz), and 8.78 (s, 1H, N*H*); ^{13}C-NMR ($CDCl_3$, 50.3 MHz): d 27.7 (CH$_2$-Lev), 29.6 (*Me*-Lev), 37.7 (CH$_2$-Lev), 41.3 ($C_{2'}$), 63.7 ($C_{5'}$), 70.6, 84.8, 87.4 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 96.8 (C_5), 127.6, 128.7 (C_o+C_m), 132.8 (C_i), 133.0 (C_p), 144.2 (C_6), 155.1 (C_2), 162.4 (C_4), 166.7 (PhC=O), 172.6 (C=O), and 206.8 (C=O); MS (ESI$^+$, m/z): 430 [(M+H)$^+$, 20%], 452 [(M+Na)$^+$, 65], and 468 [(M+K)$^+$, 40].

N-Benzoyl-5'-*O*-levulinyl-2'-deoxyadenosine (4e). R_f (10% $MeOH/CH_2Cl_2$): 0.50; Mp: 69-71 °C; IR (KBr): υ 3413, 2959, 2928, 1726, 1637, and 1616 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 300 MHz): d 2.30 (s, 3H, *Me*-Lev), 2.70 (m, 3H, CH_2-Lev+1$H_{2'}$), 2.92 (m, 2H, CH_2-Lev), 3.15 (m, 1H, $H_{2'}$), 4.40 (m, 1H, $H_{4'}$), 4.52 (m, 2H, 2$H_{5'}$), 4.85 (m, 1H, $H_{3'}$), 6.75 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 6.2 Hz), 7.80 (m, 3H, 2H_m+H_p), 8.30 (m, 2H, 2H_o), 8.78 (s, 1H, H_2 or H_8), and 8.92 (s, 1H, H_8 or H_2), ^{13}C-NMR (MeOH-d$_4$, 75.5 MHz): d 29.0 (CH_2-Lev), 29.9 (*Me*-Lev), 38.9, 40.8 (CH_2-Lev+$C_{2'}$), 65.3 ($C_{5'}$), 72.6, 86.51, 86.54 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 125.7 (C_5), 129.7, 130.0 (C_o+C_m), 134.2(C_p), 135.2 (C_i), 144.6 (C_8), 151.4 (C_4), 153.3(C_6), 153.5 (C_2), 168.4 (Ph*C*=O), 174.5 (*C*=O), and 209.7 (*C*=O); MS (ESI$^+$, *m/z*): 476 [$(M+Na)^+$, 100%] and 492 [$(M+K)^+$, 53].

N-Isobutyryl-3'-*O*-levulinyl-2'-deoxyguanosine (4g). R_f (20% $MeOH/CH_2Cl_2$): 0.60; Mp: 45-47 °C; IR (KBr): υ 3415, 2930, 1720, and 1685 cm^{-1}; ^{1}H-NMR (MeOH-d_4, 200 MHz): d 1.41 (d, 6H, *Me*-^{i}Bu, $^3J_{HH}$ 6.8 Hz), 2.33 (s, 3H, *Me*-Lev), 2.59–3.07 (m, 7H, 2CH_2-Lev+2$H_{2'}$+*CH*-^{i}Bu), 4.32 (m, 1H, $H_{4'}$), 4.50 (m, 2H, $H_{5'}$), 4.75 (m, 1H, $H_{3'}$), 6.50 (apparent t, 1H, $H_{1'}$, $^3J_{HH}$ 6.4 Hz), and 8.32 (s, 1H, H_8); ^{13}C-NMR (MeOH-d$_4$, 75.5 MHz): d 19.7 (*Me*-^{i}Bu), 29.0 (CH_2-Lev), 29.9 (*Me*-Lev), 37.2 (*C*H-^{i}Bu), 38.9, 41.1 ($C_{2'}$+CH_2-Lev), 65.3 ($C_{5'}$), 72.6, 86.1, 86.5 ($C_{1'}$+$C_{3'}$+$C_{4'}$), 121.8 (C_5), 139.8 (C_8), 150.0, 150.5 (C_2+C_4), 157.8 (C_6), 174.5 (*C*=O), 182.0 (^{i}Bu-*C*=O), and 209.7 (*C*=O); MS (ESI$^+$, *m/z*): 436 [$(M+H)^+$, 20%], 458 [$(M+Na)^+$, 100], and 474 [$(M+K)^+$, 50].

General Procedure for the enzymatic hydrolysis of thymidine tetramer bearing levulinyl protecting groups at each of the 3',-O and 5'-O terminal positions

To a solution of diprotected tetramer in 1,4-dioxane is added 0.15M phosphate buffer pH= 7 and the corresponding lipase [ratio of tetramer:enzyme is 1:1 (w/w) for CAL-A or CAL-B, and 1:3 (w/w) for PSL-C]. The mixture is allowed to react at 250 rpm for 62 h at 40°. The reactions are monitored by TLC (10% $MeOH/CH_2Cl_2$). The enzyme is filtered off and washed with CH_2Cl_2, the solvents are distilled under vacuum, and the residue is taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic layers are dried over Na_2SO_4 and evaporated to give monoacylpolynucleotides.

General Procedure for the Regioselective Enzymatic Acylation of 2'-Deoxynucleosides. The general procedure for enzymatic acylation of 2'-deoxynucleosides is shown in Figure 5. A suspension of 1 (0.2 mmol), the oxime ester

(0.6 mmol), and the lipase in dry THF (1 mL) under nitrogen was stirred at 250 rpm for the time and at the temperature indicated in Table 3. The reactions were monitored by TLC (10% MeOH/CH_2Cl_2). The enzyme was filtered off and washed with CH_2Cl_2, the solvents were distilled under vacuum, and the residue was taken up in $NaHCO_3$ (aq) and extracted with CH_2Cl_2. The combined organic layers were dried over Na_2SO_4 and evaporated. The residue was precipitated in diethyl ether to afford after filtration the monolevulinyl nucleosides 3 or 4. No further purification was necessary except in entries 10 and 11 to separate the traces of other acyl derivatives.

General Procedure for Regioselective Enzymatic Acylation of 2'-Modified Ribonucleosides. The general procedure for enzymatic acylation of 2'-modified ribonucleosides is shown in Figure 7. A 2'-modified ribonucleoside 5 was reacted with an oxime levulinyl ester in THF in the presences of lipase (CAL-B or PSL-C). The resulting product 8 (CAL-B) is acylated at the 5'-position and 9 (PSL-C) is acylated at the 3'-position.

In view of the foregoing, it can be seen that the present invention provides facile methodologies for selectively preparing 3'-*O*-levulinyl nucleosides and 5'-*O*-levulinyl nucleosides from nucleoside and/or 3', 5'-di-O-levulinyl nucleoside precursors. The present invention is adaptable to large-scale preparation of protected nucleosides. Compounds prepared by the inventive methods are useful in a variety methods for preparation of oligonucleotides.

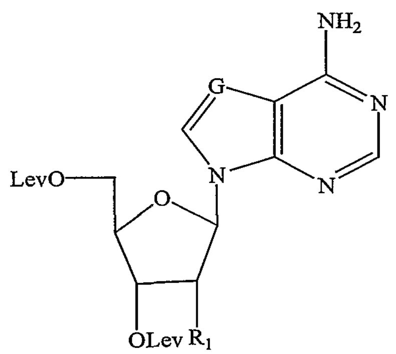
While the present invention has been illustrated by reference to certain embodiments that may at present be preferred, the methodologies disclosed herein are general and may be applied to a variety of substrates, and in particular to substrates having hydroxyl groups thereon. The person having skill in the art will therefore recognize that other embodiments are within the scope of the invention as generally described herein. No reference to any particular embodiments herein is intended to imply any limitation to the scope of the present invention.

All references cited herein are expressly incorporated herein by reference in their entirety.

What is claimed is:

1. A method for the selective deprotection of a 3', 5'-di-O-levulinyl nucleoside comprising
   a. selecting a hydrolase effective to direct regioselective hydrolysis of one of said levulinyl positions of the nucleoside; and
   b. contacting the 3', 5'-di-*O*-levulinyl nucleoside with said hydrolase for a time and under conditions effective to yield the corresponding 3'-*O*-levulinyl and 5'-*O*-levulinyl nucleoside.
2. The method of claim 1, wherein said hydrolase is a lipase.
3. The method of claim 2 wherein said lipase is CAL-A, CAL-B, PSL-C, porcine pancreatic lipase, *Chromobacteriaum viscosum* lipase, *Mucor miehei* lipase, *Humicola lanuginosa* lipase, *Penicillium camemberti* lipase, or *Candida rugosa* lipase.
4. The method of claim 3 wherein said lipase is CAL-A.
5. The method of claim 3 wherein said lipase is CAL-B.
6. The method of claim 3 wherein said lipase is PSL-C.
7. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 5'-*O*-levulinyl position comprising selecting a hydrolase effective to direct regioselective hydrolysis of said 3', 5'-di-*O*-levulinyl nucleoside at the 5'-*O* -levulinyl position and contacting said 3', 5'-di-*O*-levulinyl nucleoside with said hydrolase for a time and under conditions effective to yield a 3'-*O*-levulinyl nucleoside.
8. The method of claim 7, wherein said hydrolase is a lipase.
9. The method of claim 8 wherein said lipase is CAL-B.
10. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position comprising selecting a hydrolase effective to direct regioselective hydrolysis of said 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O* -levulinyl position and contacting said 3', 5'-di-*O*-levulinyl nucleoside with said hydrolase for a time and under conditions effective to yield a 5'-*O*-levulinyl nucleoside.
11. The method of claim 10, wherein said hydrolase is a lipase.
12. The method of claim 11 wherein said lipase is CAL-A.
13. The method of claim 11 wherein said lipase is PSL-C.
14. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 5'-*O*-levulinyl position comprising selecting a hydrolase effective to direct regioselective

hydrolysis of said 3', 5'-di-*O*-levulinyl nucleoside at the 5'-*O* -levulinyl position and contacting said 3', 5'-di-*O*-levulinyl nucleoside with said hydrolase for a time and under conditions effective to yield a 3'-*O*-levulinyl nucleoside wherein said 3', 5'-di-*O*-levulinyl nucleoside has one of the following formulas:



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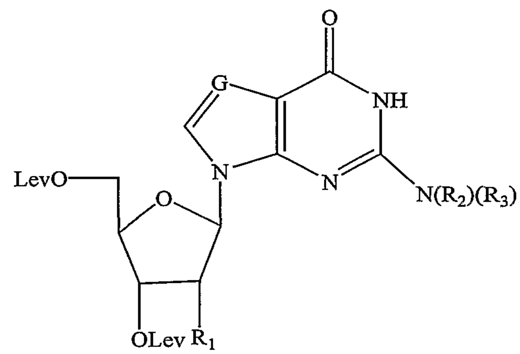

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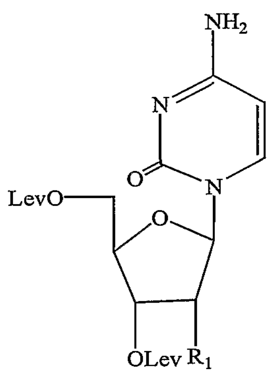

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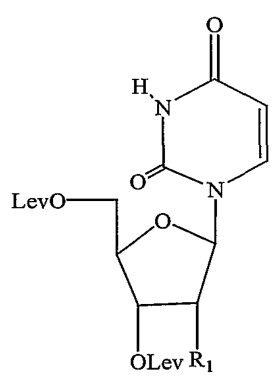

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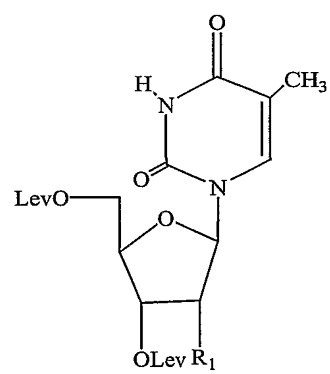


V

wherein:

R_1 is -H, -hydroxyl, a protected hydroxyl, or a 2'-substituent; and

R_2 and R_3 are, independently, -H or an amino protecting group;

G is N or CH; and

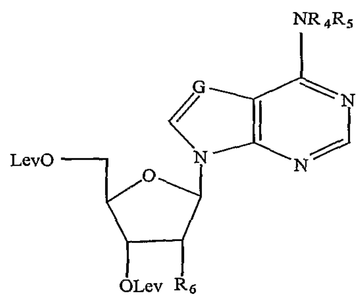
Lev is -C(O)-$(CH_2)_2$-C(O)-CH_3.

15. The method of claim 14, wherein said hydrolase is a lipase.

16. The method of claim 15 wherein said lipase is CAL-B.

17. The method of claim 14 wherein said 3',5'-di-*O*-levulinyl nucleoside is an adenosine, cytosine, thymidine, or an *N*-isobutyl guanosine.

18. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position comprising selecting a hydrolase effective to direct regioselective hydrolysis of said 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O* -levulinyl position and contacting said 3', 5'-di-*O*-levulinyl nucleoside with said hydrolase for a time and under conditions effective to yield a 5'-*O*-levulinyl nucleoside wherein said 3', 5'-di-*O*-levulinyl nucleoside has one of the following formulas:



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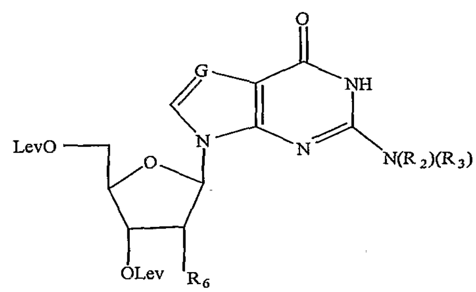

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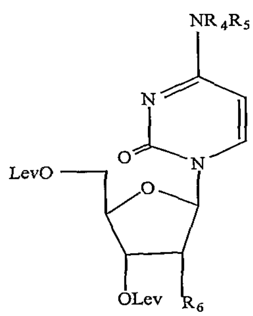

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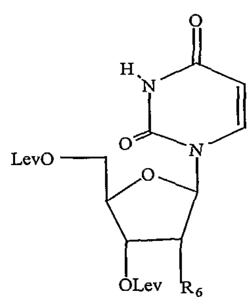
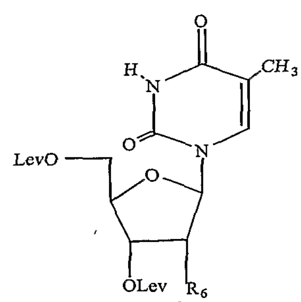


IX



X

wherein:

R_6 is -H, or -OH;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$.

19. The method of claim 18, wherein said hydrolase is a lipase.

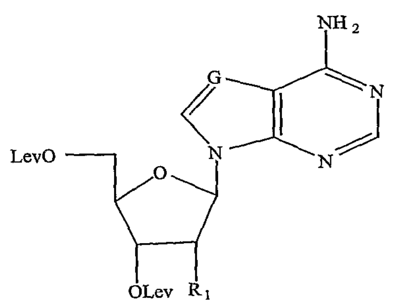
20. The method of claim 19 wherein said lipase is CAL-A.

21. The method of claim 19 wherein said lipase is PSL-C.

22. The method of claim 18 wherein said 3',5'-di-*O*-levulinyl nucleoside is 3',5'-di-*O*-levulinyl thymidine, 3',5'-di-*O*-levulinyl cytosine, or 3',5'-di-*O*-levulinyl *N*-benzoyl adenosine.

23. The method of claim 22 wherein said 3',5'-di-*O*-levulinyl nucleoside is *N*-isobutylguanosine.

24. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 5'-*O* levulinyl position wherein said 3', 5'-di-*O*-levulinyl nucleoside has one of the following formulas:



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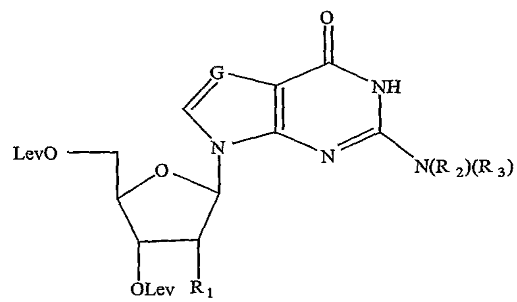

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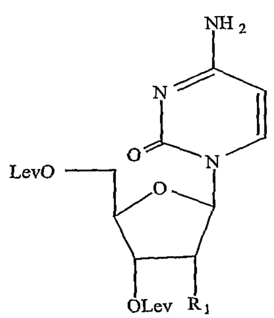

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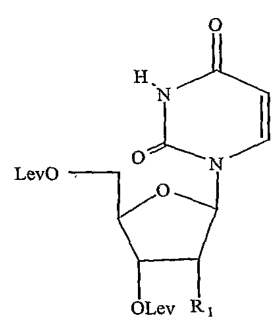

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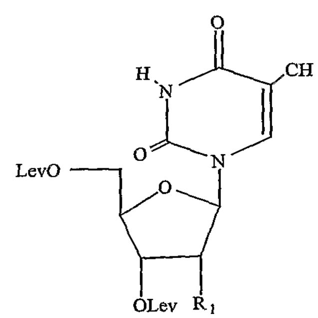


V

wherein:

R_1 is -H, -hydroxyl, a protected hydroxyl, or a 2'-substituent; and

R_2 and R_3 are, independently, -H or an amino protecting group;

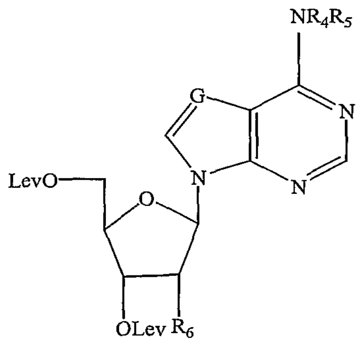
G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$;

comprising contacting said 3', 5'-di-*O*-levulinyl nucleoside with CAL-B for a time and under conditions effective to hydrolyze said 3', 5'-di-*O*-levulinyl nucleoside at the 5'-*O*-levulinyl position.

25. The method of claim 24 wherein said 3'-,5'-di-*O*-levulinylnucleoside comprises an adenosine, cytosine, thymidine, or an *N*-isobutyl guanosine moiety.

26. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position wherein said 3', 5'-di-*O*-levulinyl nucleoside has one of the following formulas:



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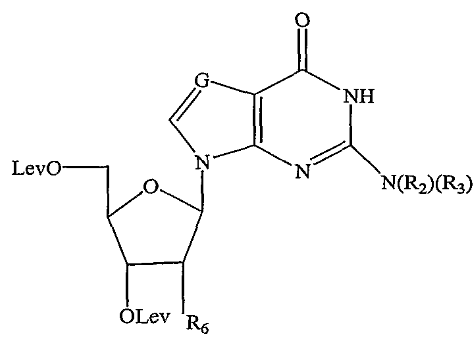

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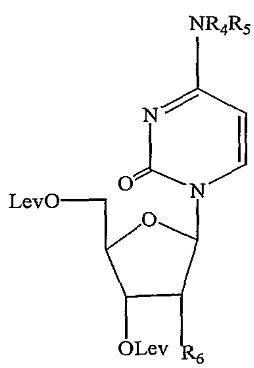

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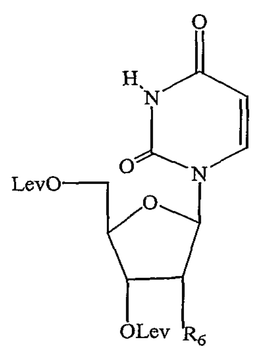

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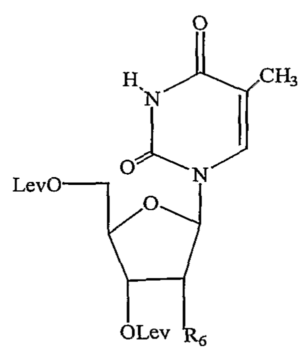


X

wherein:

R_6 is -H or -hydroxyl;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

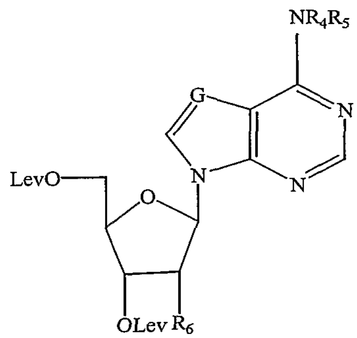
G is N or CH; and

Lev is -C(O)-$(CH_2)_2$-C(O)-CH_3;

comprising contacting said 3', 5'-di-*O*-levulinyl nucleoside with PSL-C for a time and under conditions effective to hydrolyze said 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position.

27. The method of claim 26 wherein said 3'-,5'-di-*O*-levulinyl nucleoside comprises an *N*-isobutylguanosine moiety.

28. A method for the selective deprotection of a 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position wherein 3', 5'-di-*O*-levulinyl nucleoside has one of the following formulas:



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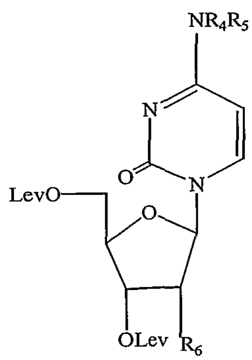

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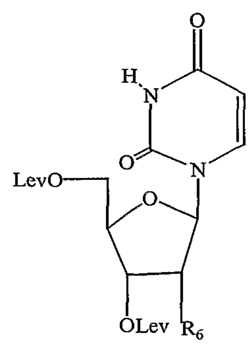

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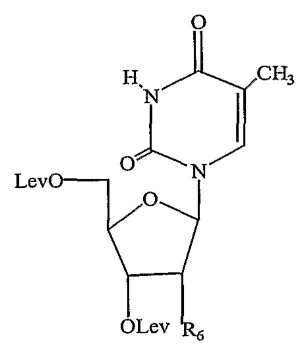


X

wherein:

R_6 is -H or -OH;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$;

comprising contacting said 3', 5'-di-*O*-levulinyl nucleoside with CAL-A for a time and under conditions effective to hydrolyze said 3', 5'-di-*O*-levulinyl nucleoside at the 3'-*O*-levulinyl position.

29. The method of claim 28 wherein said 3',5'-di-*O*-levulinyl nucleoside comprises a thymidine, cytosine, or *N*-benzoyl adenosine moiety.

30. A method for protecting a hydroxyl moiety of a nucleic acid at at least one of a 2'-O, 3'-O, or 5'-O position comprising reacting said nucleic acid with levulinic **acid in the** presence of a coupling agent that is attached to a polymeric support for a time and under conditions effective to form an ester at said 2'-O, 3'-O or 5'-O position.

31. The method of claim 30, wherein said nucleic acid is a nucleoside.

32. The method of claim 30, wherein said coupling agent is a carbodiimide.

33. The method of claim 32, wherein said carbodiimide is cyclohexylcarbodiimide.

34. The method of claim 30, wherein said polymeric support is a polystyrene.

35. The method of claim 30, wherein said polymeric support is a polyethylene glycol.

36. A method for acylating at least one hydroxyl moiety of a carbohydrate comprising reacting said carbohydrate with levulinic acid in the presence of a coupling agent that is attached to a polymeric support for a time and under conditions effective to form an ester.

37. The method of claim 36, wherein said coupling agent is a carbodiimide.

38. The method of claim 37, wherein said carbodiimide is cyclohexylcarbodiimide.

39. The method of claim 36, wherein said polymeric support is a polystyrene support.

40. The method of claim 36, wherein said polymeric support is a polyethylene glycol support.

41. A method for acylating at least one hydroxyl moiety of a steroid molecule comprising reacting said steroid molecule with levulinic acid in the presence of a coupling agent that is attached to a polymeric support for a time and under conditions effective to form an ester.

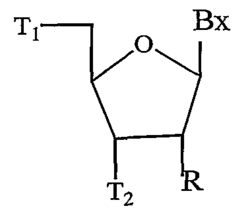
42. The method of claim 41, wherein said coupling agent is a carbodiimide.

43. The method of claim 42, wherein said carbodiimide is cyclohexylcarbodiimide.

44. The method of claim 41, wherein said polymeric support is a polystyrene support.

45. The method of claim 41, wherein said polymeric support is a polyethylene glycol support.

46. A method for protecting a hydroxyl moiety on a compound having the following formula:



wherein:

B_X is a nucleobase;

T_1 and T_2, independently, are OH, a hydroxyl protecting group, an activated phosphate group, a nucleotide, a nucleoside, or an oligonucleotide;

R is -H, -hydroxyl, a protected hydroxyl or a 2' substituent group;

provided that at least one of T_1, T_2 or R is -OH;

comprising reacting said compound with levulinic acid in the presence of a coupling agent that is attached to a solid support for a time and under conditions effective to form an ester between said hydroxyl moiety and the levulinyl group.

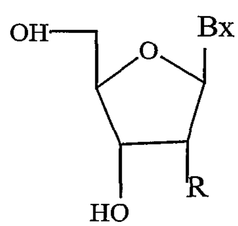
47. The method of claim 46, wherein said coupling agent is a carbodiimide.

48. The method of claim 47, wherein said carbodiimide is a cyclohexylcarbodiimide.

49. The method of claim 46, wherein said polymeric support is a polystyrene support.

50. The method of claim 46, wherein said polymeric support is a polyethyleneglycol support.

51. A method for protecting the 3'-O and 5'-O positions of a compound having the following formula:

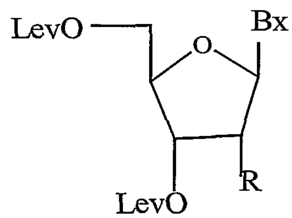


wherein:

B_X is a nucleobase; and

R is –H, or a 2' – substituent;

comprising reacting said compound with levulinic acid in the presence of a coupling agent that is attached to a solid support for a time and under conditions effective to form a compound having formula:

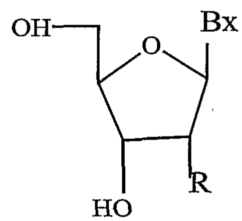


wherein Lev is a -levulinyl.

52. The method of claim 51 wherein said coupling agent attached to a polymeric support is cyclohexylcarbodiimide attached to a polymeric support.

53. The method of claim 52 wherein said polymeric support is a polystyrene polymeric support.

54. A method for protecting the 3'-O and 5'-O positions of a compound having the following formula:

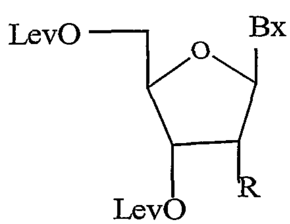


wherein:

Bx is a nucleobase; and

R is –H, or a 2'- substituent;

comprising reacting said compound with levulinic acid in the presence of cyclohexylcarbodiimide that is attached to a polystyrene polymeric support for a time and under conditions effective to form a compound having the following formula:



wherein Lev is -levulinyl.

55. A method for acylating a hydroxyl moiety comprising reacting said hydroxyl moiety with levulinic acid in the presence of a coupling agent that is attached to a polymeric support for a time and under conditions effective to yield an ester.

56. The method of claim 55 wherein said coupling agent is a carbodiimide

57. The method of claim 56 wherein said carbodiimide is cyclohexylcarbodiimide.

58. The method of claim 55 wherein said polymeric support is a polystyrene.

59. The method of claim 55 wherein said polymeric support is polyethylene glycol.

60. A method for generating a cyclohexylcarbodiimide derivatized polymeric support from a cyclohexylurea derivatized polymeric support comprising reacting said cyclohexylurea derivatized polymeric support with a dehydrating agent in an organic solvent for a time and under conditions effective to yield said cyclohexylcarbodiimide derivatized polymeric support.

61. The method of claim 60 wherein said dehydrating agent is $POCl_3$.

62. The method of claim 61 wherein said dehydrating agent is tosyl chloride.

63. The method of claim 62 wherein said organic solvent is CH_2Cl_2, $CHCl_3$, hexane, or pyridine.

64. The method of claim 61 wherein said polymeric support is a polystyrene polymeric support.

65. A method for generating a cyclohexylcarbodiimide derivatized polymeric support from a cyclohexylurea derivatized polymeric support comprising the steps of:

    a. reacting said cyclohexylurea derivatized polymer support with a dehydrating agent in an organic solvent for a time and under conditions effective to form a salt; and

    b. contacting said salt with an aqueous solution to form said cyclohexylcarbodiimide derivatized polymeric support.

66. The method of claim 65 wherein said dehydrating agent is $POCl_3$.

67. The method of claim 65 wherein said dehydrating agent is tosyl chloride.

68. The method of claim 65 wherein said organic solvent is CH_2Cl_2, $CHCl_3$, hexane, or pyridine.

69. The method of claim 65 wherein said polymeric support is a polystyrene polymeric support.

70. A method for the selective acylation of a nucleoside comprising:

    a. selecting a hydrolase effective to direct regioselective acylation of one of the 5'- or 3'- hydroxyl positions of the nucleoside; and

    b. contacting the nucleoside with an acylating agent in the presence of said hydrolase for a time and under conditions effective to yield an acylated

nucleoside selected from a 3'-*O*-levulinyl nucleoside and a 5'-*O*-levulinyl nucleoside.

71. The method of claim 70, wherein said hydrolase is a lipase.

72. The method of claim 71, wherein said lipase is CAL-A, CAL-B, PSL-C, porcine pancreatic lipase, *Chromobacteriaum viscosum* lipase, *Mucor miehei* lipase, *Humicola lanuginosa* lipase, *Penicillium camemberti* lipase, or *Candida rugosa* lipase.

73. The method of claim 72 wherein said lipase is CAL-A.

74. The method of claim 72 wherein said lipase is CAL-B.

75. The method of claim 72 wherein said lipase is PSL-C.

76. A method for the selective acylation of a nucleoside at the 5'- position comprising selecting a hydrolase effective to direct regioselective acylation of said nucleoside at the 5'- position and contacting said nucleoside with an acylating agent in the presence of said hydrolase for a time and under conditions effective to yield a 5'-*O*-levulinyl nucleoside.

77. The method of claim 76, wherein said hydrolase is a lipase.

78. The method of claim 77 wherein said lipase is CAL-B.

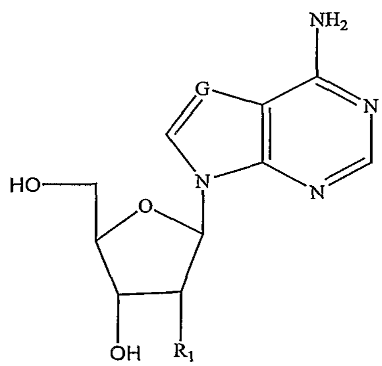
79. A method for the selective acylation of a nucleoside at the 3'- position comprising selecting a hydrolase effective to direct regioselective hydrolysis of said nucleoside at the 3'- position and contacting said nucleoside with an acylating agent in the presences of said hydrolase for a time and under conditions effective to yield a 3'-*O*-levulinyl nucleoside.

80. The method of claim 79, wherein said hydrolase is a lipase.

81. The method of claim 80 wherein said lipase is CAL-A.

82. The method of claim 80 wherein said lipase is PSL-C.

83. A method for the selective acylation of a nucleoside at the 5'- position, comprising selecting a hydrolase effective to direct regioselective acylation of said nucleoside at the 5'- position and contacting said nucleoside with an acylating agent in the presence of said hydrolase for a time and under conditions effective to yield a 5'-*O*-levulinyl nucleoside wherein said nucleoside has one of the following formulas:



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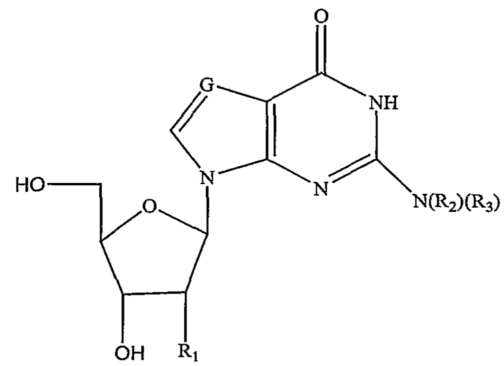

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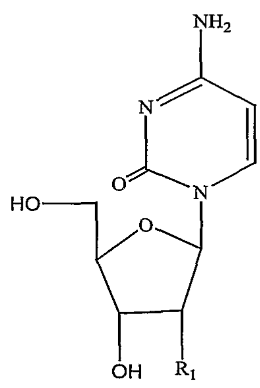

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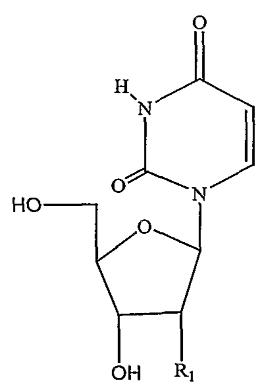

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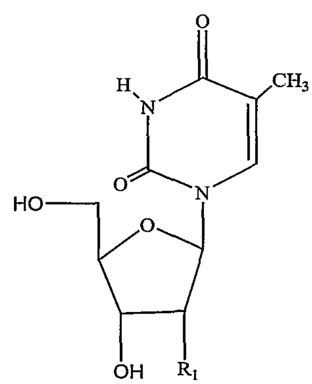


V

wherein:

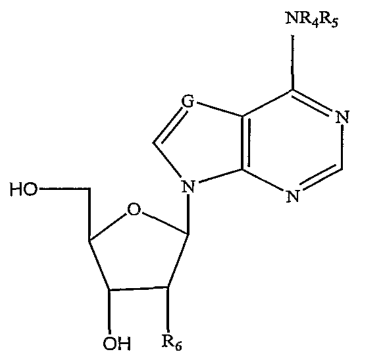
R_1 is -H, -hydroxyl, a protected hydroxyl, or a 2'-substituent; and
R_2 and R_3 are, independently, -H or an amino protecting group; and
G is N or CH.

84. The method of claim 83, wherein said hydrolase is a lipase.

85. The method of claim 84, wherein said lipase is CAL-B.

86. The method of claim 83 wherein said nucleoside is an adenosine, cytosine, thymidine, or an *N*-isobutyl guanosine.

87. A method for the selective acylation of a nucleoside at the 3'-position, comprising selecting a hydrolase effective to direct regioselective acylation of said nucleoside at the 3'- position and contacting said nucleoside with an acylating agent in the presence of said hydrolase for a time and under conditions effective to yield a 3'-*O*-levulinyl nucleoside, wherein said nucleoside has one of the following formulas:



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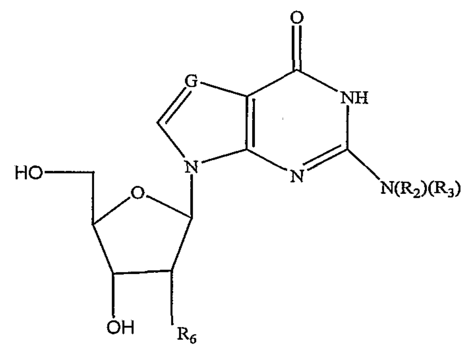

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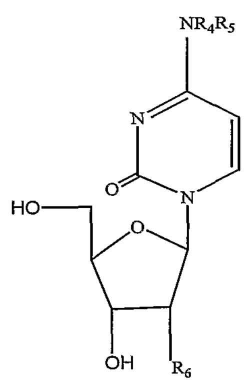

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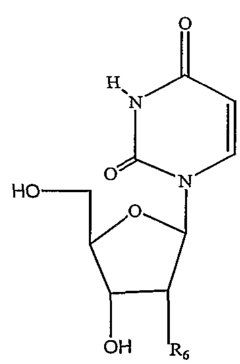

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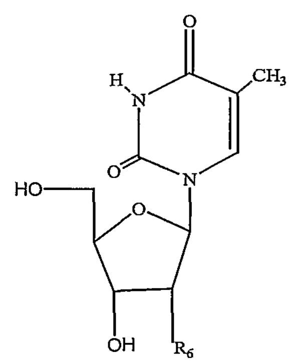


X

wherein:

R_6 is -H, or -OH;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$.

88. The method of claim 87, wherein said hydrolase is a lipase.

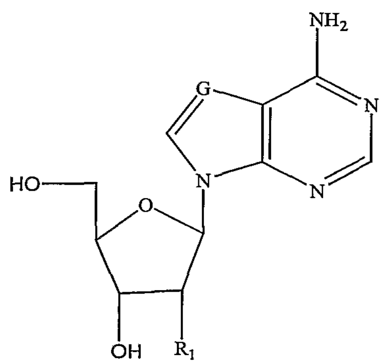
89. The method of claim 88, wherein said lipase is CAL-A.

90. The method of claim 88, wherein said lipase is PSL-C.

91. The method of claim 87, wherein said nucleoside is thymidine, cytosine, or *N*-benzoyl adenosine.

92. The method of claim 91 wherein said nucleoside is *N*-isobutylguanosine.

93. A method for the selective acylation of a nucleoside at the 5'- position wherein said nucleoside has one of the following formulas:



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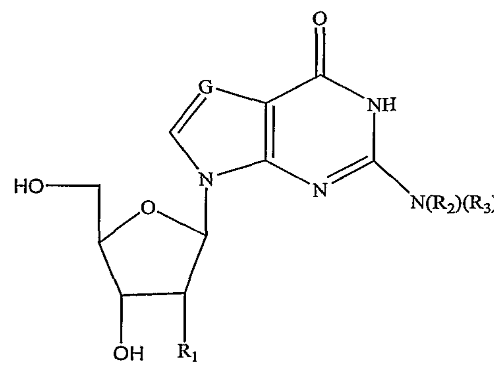

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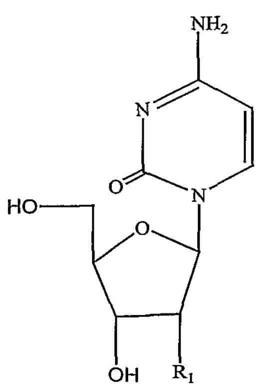

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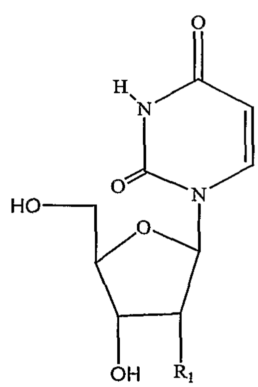

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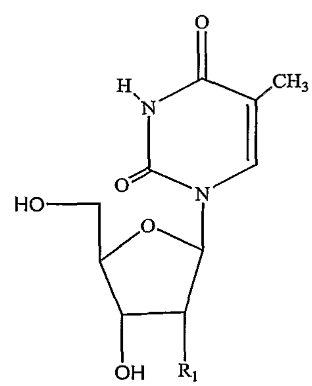


V

,

wherein:

R_1 is -H, -hydroxyl, a protected hydroxyl, or a 2'-substituent; and

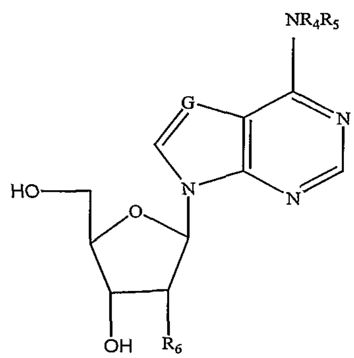
R_2 and R_3 are, independently, -H or an amino protecting group; and

G is N or CH;

said method comprising contacting said nucleoside with an acylating agent and CAL-B for a time and under conditions effective to acylated said nucleoside at the 5'-position.

94. The method of claim 93 wherein said nucleoside comprises an adenosine, cytosine, thymidine, or an *N*-isobutyl guanosine moiety.

95. A method for the selective acylation of a nucleoside at the 3'-position wherein said nucleoside has one of the following formulas:



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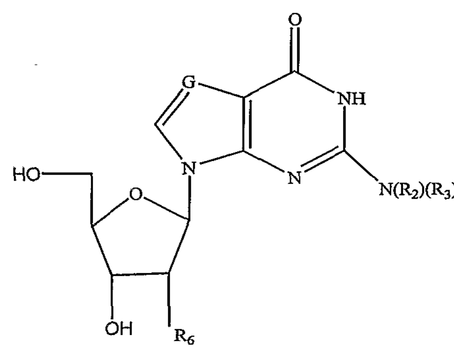

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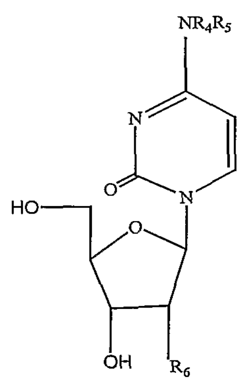

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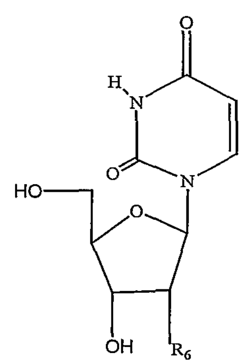

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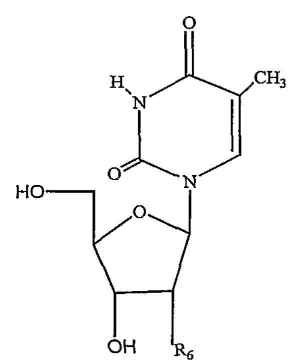


X

wherein:

R_6 is -H or -hydroxyl;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group;

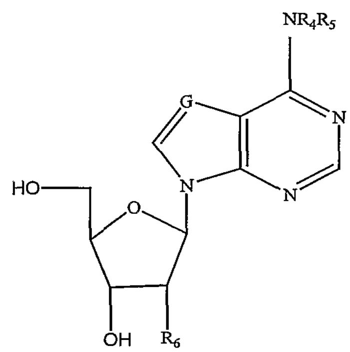
G is N or CH; and

Lev is $-C(O)-(CH_2)_2-C(O)-CH_3$;

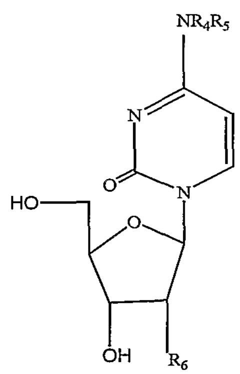
comprising contacting said nucleoside with an acylating agent in the presence of PSL-C for a time and under conditions effective to acylate said nucleoside at the 3'- position.

96. The method of claim 95 wherein said nucleoside comprises an *N*-isobutylguanosine moiety.

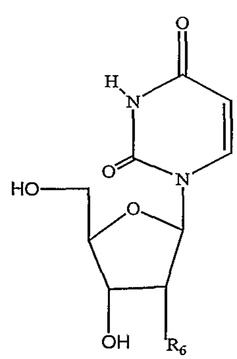
97. A method for the selective acylation of a nucleoside at the 3'- position, wherein said nucleoside has one of the following formulas:



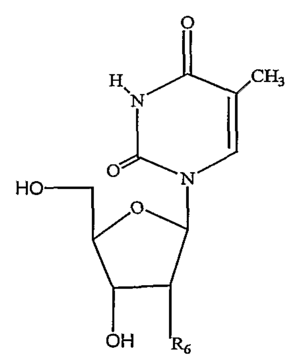
VI



VIII



IX



X

wherein:

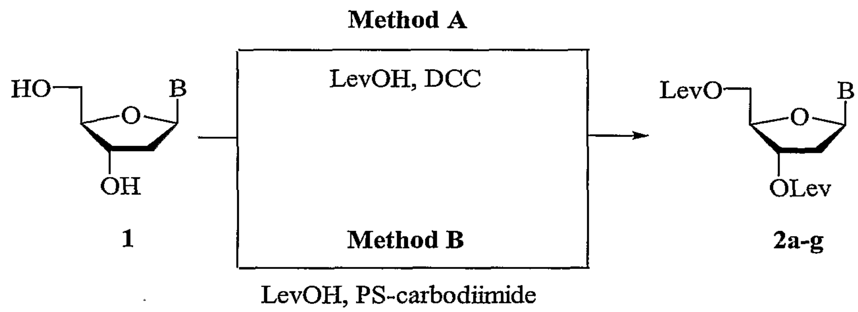
R_6 is -H or -OH;

R_2, R_3, R_4, and R_5 are each, independently, -H or an amino protecting group; and

G is N or CH;

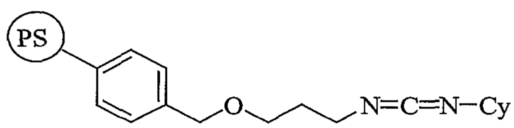
the method comprising contacting said nucleoside with CAL-A for a time and under conditions effective to acylate said nucleoside at the 3'- position.

98. The method of claim 97 wherein said nucleoside comprises a thymidine, cytosine, or *N*-benzoyl adenosine moiety.



a, B= T; **b**, B= C; **c**, B= C^{Bz}; **d**, B= A; **e**, B= A^{Bz}; **f**, B= G; **g**, B= G^{iBu}

Method A: LevOH, DCC, DMAP, Et_3N, 1,4-Dioxane.
Method B: LevOH, PS-carbodiimide, DMAP, DMAP•HCl, Et_3N, 1,4-Dioxane.



PS-carbodiimide

LevOH=

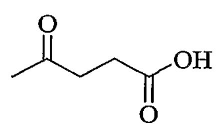
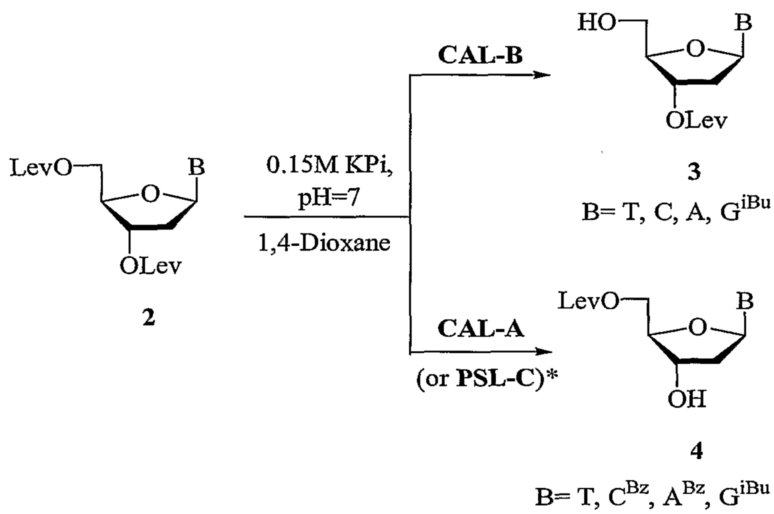
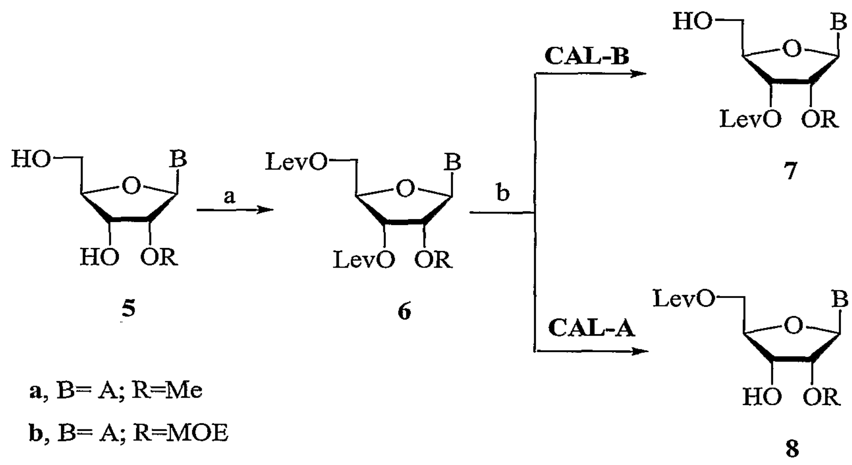


FIGURE 1



***PSL-C** was used for di-Lev-dG^{iBu} (**2g**) since **CAL-A** did not catalyze the hydrolysis.

FIGURE 2



a, B= A; R=Me

b, B= A; R=MOE

c, B= 5-Me-C; R=Me

d, B= 5-Me-C; R=MOE

(a) LevOH, DCC, Et_3N, DMAP, 1,4-dioxane.

(b) 0.15M KPi (pH=7), 1,4-dioxane.

FIGURE 3

Table 1. **Regioselective Enzymatic Hydrolysis of di-Levulinyl esters 2.**

					Yield (%)[a]	
entry	**substrate**	**enzyme**	**T (°C)**	***t* (h)**	3	4
1	2a	**CAL-B**[b]	**40**	**62**	**85**	
2	2b	**CAL-B**	**30**	**62**	**84**	
3	2d	**CAL-B**	**40**	**28**	**98**	
4	2g	**CAL-B**[c]	**40**	**18**	**80**	
5	2a	**CAL-A**[b]	**40**	**86**		**70**
6	2c	**CAL-A**[b]	**40**	**62**		**78**
7	2e	**CAL-A**	**40**	**68**		**85**
8	2g	**PSL-C**	**60**	**28**		**93**

a **Isolated yield.**

b **An extra fraction of lipase (30 mg) was added after 30 h.**

c **It was used a ratio of 1:2 w/w (2g/CAL-B).**

a, B= T; **b**, B= C; **c**, B= C^{Bz}; **d**, B= A; **e**, B= A^{Bz}; **f**, B= G; **g**, B= G^{iBu}

FIGURE 4

Regioselective Enzymatic Acylation of 2'-deoxynucleosides

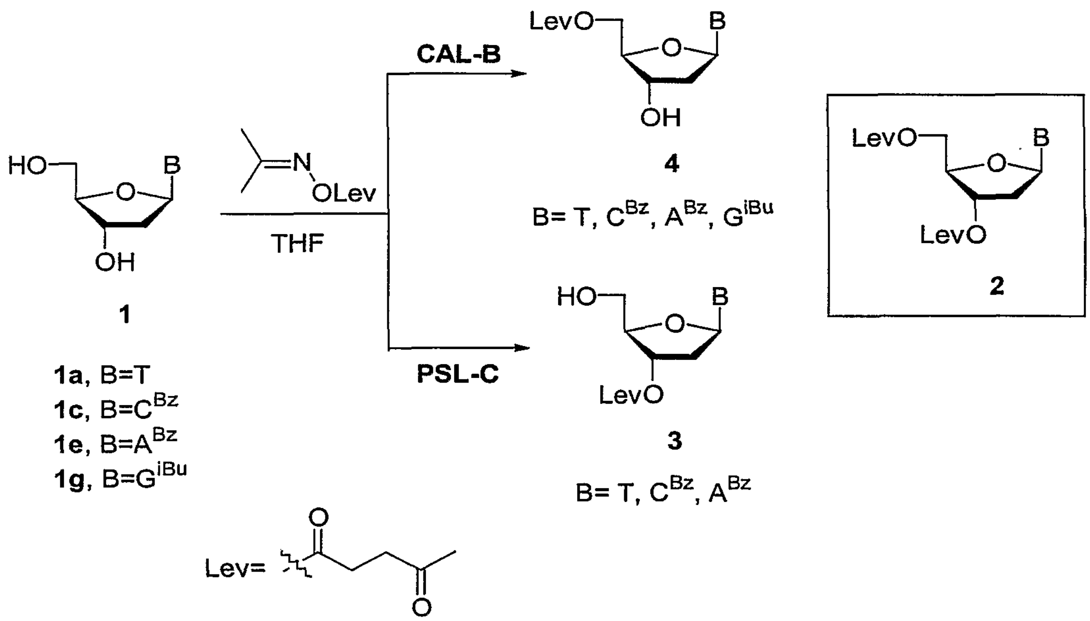


FIGURE 5

Table 3. **Regioselective Enzymatic Acylation of 1[a].**

entry	substrate	enzyme	T (°C)	*t* (h)	1	2	3[b]	4[b]
1	1a (T)	PSL-C[c]	30	22			>97 (85)	
2	1c (C^{Bz})	PSL-C[c]	30	46			>97 (77)	
3	1c (C^{Bz})	PSL-C[c]	40	46			>97 (68)	
4	1c (C^{Bz})	PSL-C[c]	60	22			>97 (73)	
5	1e (A^{Bz})	PSL-C[c]	40	24			>97 (89)	
6	1e (A^{Bz})	PSL-C[c]	60	15			>97 (75)	
7	1g (G^{iBu})	PSL-C[c]	30	27		17	20	63
8	1g (G^{iBu})	PSL-C[c]	60	14	22	31	35	12
9	1a (T)	CAL-B[d]	30	22				>97 (74)
10	1c (C^{Bz})	CAL-B[d]	30	24		2	6	92 (76)
11	1e (A^{Bz})	CAL-B[d]	30	25		1	5	94 (71)
13	1g (G^{iBu})	CAL-B[d]	30	16				>97(79)
14	1a (T)	PSL-C[e,f]	30	14			>97 (92)	
15	1c (C^{Bz})	PSL-C[e,f]	30	14			>97 (81)	
16	1e (A^{Bz})	PSL-C[e,f]	30	14			>97 (90)	

[a] Percentage of compounds calculated by ^{1}H NMR (± 3%). [b] Isolated yields are given in parenthesis. [c] Ratio 1:PSL-C is 1:3 (w/w). [d] Ratio 1:CAL-B is 1:1 (w/w). [e] Ratio 1:PSL-C is 1:2 (w/w).
[f] Scale-up to 5g of 1.

FIGURE 6

Regioselective Enzymatic Acylation of 2'-*O*-alkylribonucleosides

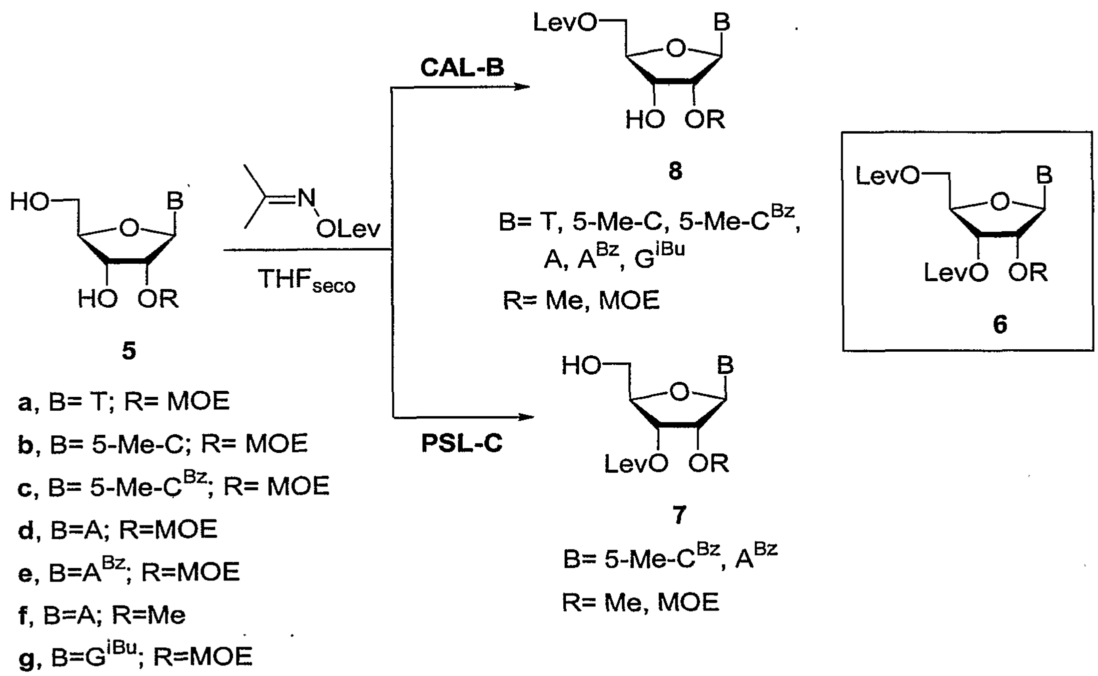


FIGURE 7

Table 4. **Regioselective Enzymatic Acylation of 5[a].**

entry	substrate	enzyme	T (°C)	*t* (h)	5	6	7[b]	8[b]
1	5a (T)	**PSL-C[c]**	**30**	**23**	**15**		**64**	21
2	5b (C)	**PSL-C[c]**	**30**	**23**	**>99**			
3	5c (C^{Bz})	**PSL-C[c]**	**30**	**2.5**			**>97** (82)	
4	5d (A)	**PSL-C[c]**	**30**	**23**	**>99**			
5	5e (A^{Bz})	**PSL-C[c]**						
6	5f (A)	**PSL-C[c]**	**30**	**23**	**23**	**3**	**66**	**8**
7	5g (G^{iBu})	**PSL-C[c]**	**30**	**172**	**68**		**32**	
8	5g (G^{iBu})	**PSL-C[c]**	**60**	**172**	**64**		**36**	
9	5a (T)	**CAL-B[d]**	**40**	**8**				**>97** (70)
10	5b (C)	**CAL-B[d]**	**40**	**2.5**				**>97** **(61)**
11	5c (C^{Bz})	**CAL-B[d]**	**40**	**5.5**				**>97** (86)
12	5d (A)	**CAL-B[d]**	**40**	**8**				**>97** (86)
13	5e(A^{Bz})	**CAL-B[d]**						**>97**
14	5f (A)	**CAL-B[d]**	**40**	**2.5**				**>97** (82)
15	5g (G^{iBu})	**CAL-B[d]**	**40**	**3.5**				**>97** (88)
16	5c (C^{Bz})	**PSL-C[e]**	**30**	**2.5**			**>97** (87)	

[a] **Percentage of compounds calculated by ^{1}H NMR (± 3%).** [b] **Isolated yields are given in parenthesis.** [c] **Ratio** 5:**PSL-C is 1:3 (w/w).** [d] **Ratio** 5:**CAL-B is 1:1 (w/w).** [e] **Scale-up to 4g of** 5.

FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/08547

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/02, 21/04 19/00; C12Q 1/68; C12P 41/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 22.1, 24.3; 435/280, 876, 921, 931, 6, 69.1,195, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
GIBCO CATALOG, ROCHE APPLIED SCIENCE CALATOG,

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,538,891 A (SCHNEIDER ET AL) 23 JULY 1996 (23/07/1996) see abstract, column 1, lines 9-61, column 8,lines 17-49, table 2.	1-29, 70-98
Y	US 5,594,117 A (UDREA ET AL) 14 JANUARY 1997 (14/01/1997) see abstract, column 2, lines 16-59, column 3, lines 36-68, column 4, lines 1-60, column 13, lines 6-67, also see example 1.	30-40, 55-69
Y	WO 00/66605 A2 (CYCLOPS GENOMES SCIENCES LIMITED) 09 NOVEMBER 2000 (09/11/2000) see abstract, page 19-24 ,all lines, column 3, page 49-52, all lines , also see example 13, 24, and figures 1-9.	1-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 JULY 2002	

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Dorthea Lawrence for CHRISTINE MAUPIN
Facsimile No. (703) 305-3230	Telephone No. (703) -308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/08547

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/08547

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FERRERO et al. Biocatalytic Selective Modifications of Conventional Nucleosides, Carbocyclic Nucleoside, and C-nucleosides. Chem. Rev. 10 November 2000, Vol. 100, pages 4319-4347, see pages 4320-4329.	36-40, 55-59.
A	US 6,222,030 B1 (DELLINGER ET AL) 24 APRIL 2001 (24/04/2001) see abstract, column 6-8, also see figures 1-5.	1-29,

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/08547

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.1, 22.1, 24.3; 435/280, 876, 921, 931, 6, 69.1,195, 320.1

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, USPATFULL, CAPLUS, MEDLINE
search terms: nucleoside, modification, esterification, ethers, support, polymer, lipase, hydrolase, hydroxylate, subsitution, steroid, anchored

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-29, drawn to methods of selective deprotection with a regioselective hydrolase.
Group II, claim(s) 30-35,and 46-54, drawn to methods of protecting a hydroxyl moiety of a nucleic acid.
Group III, claim(s) 36-40, and 70-98, drawn to methods of acylation of hydroxyl moiety of a carbohydrate.
Group IV, claim(s)41-45 drawn to method of acylating a hydroxyl moiety of a steroid.
Group V, claim(s)55-59, drawn to methods of acylating any hydroxyl moiety.
Group VI, claim(s)60-69, drawn to methods of making a cyclohexylcarbodiimide derivatized polymeric support.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions listed as Groups I-VI do not have a single inventive concept or contain a special feature that would make the claims united under a single inventive concept. Groups I and III use lipases for the direction of regioselection, there is at least one article that anticipates the use of the lipases of Groups I and Group III Schneider et al, US Patent No. 5,538,891 issued 23 July 1996. Groups II, IV, V, and VI does not use the lipases of Group I and Group III. The modification of Groups II, IV, V, and VI lack a single inventive entity because there is at least one article that anticipates the modification of nucleic acids and nucleic acids supported by a solid support US Patent 5,94,117 issued 14 January 1997.